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Mass Spectrometry Discovery-Based Proteomics to Examine Anti-Aging Effects of the Nutraceutical NT-020 in Rat Serum

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Medical Sciences with a concentration in Neuroscience

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Dedication

I dedicate this work to my family, Alan, Candace, Carolyn, and Jimmy, my brother, Patrick, and my partner, Will. Without your unfailing support and encouragement, this would not have been possible. Thank you for your love and support throughout this process.
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Abstract

Aging is a complex physiological process that leads to the deterioration of all cells and tissues throughout the body. Aging is a major risk factor for the onset of many degenerative diseases in both the central nervous system (CNS) and the periphery, but even nonpathological aging (“normal” aging) is associated with chronic inflammation, oxidative stress, and decreased stem cell proliferation and regenerative capacity. This decreased regenerative capacity in stem cell niches is thought to be a key component underlying the aging process and many disease states associated with aging.

While the exact biological mechanisms underlying impaired stem cell proliferation and regeneration with age remain unclear, two areas of investigation are being explored: cell-autonomous changes and cell non-autonomous changes. Cell autonomous changes involve the senescence of genes such as p53, p16\textsuperscript{INK4A}, critical proteins involved in canonical pathways such as the Wnt/\(\beta\)-catenin pathway, and others. Cell non-autonomous effects on stem cell niches refers to various circulating factors, such as pro-inflammatory cytokines, chemokines, and other proteins that may influence the microenvironment.

Highly relevant early examples of circulating factors impacting stem cell proliferation come from heterochronic parabiosis studies. Heterochronic parabiosis entails the surgical union of the circulatory systems of a young mouse and an old mouse. It was observed that exposure to blood from old mice had a deleterious impact on stem cell proliferation in young mice, however blood from young mice had a positive effect on old mice. Similarly, when young mice were injected with plasma from old mice, their performance on cognitive behavioral paradigms such as contextual fear conditioning and the radial arm water maze was impaired, thus
underscoring the fact that circulating factors in the aging systemic milieu can impact the CNS. While parabiosis studies have proven useful for the study of circulating factors, they are not highly translational. We and others have found that nutraceutical intervention is able to reverse some of the age-related decline in the CNS observed in rats and human subjects. In particular, we have demonstrated that a particular nutraceutical called NT-020, a proprietary blend of blueberries, green tea, vitamin D3, and carnosine, is able to rescue age-related cognitive decline, reverse impaired neurogenesis, and exert anti-inflammatory effects. In vitro, it has been previously demonstrated that rat hippocampal neural progenitor cells (NPCs) and bone marrow-derived mesenchymal stem cells (MSCs) cultured with serum from aged rats showed decreased proliferation as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-
dephtyltetrazolium bromide (MTT) and 5-bromo-2'-deoxyuridine (BrdU) assays. However, when NPCs and MSCs were cultured with serum from aged rats given a diet supplemented with NT-020, the proliferation rates were not different from those of cells cultured with serum from young rats.

In studies with rats maintained on a standard diet or an NT-020-supplemented diet for one month, aged rats showed delayed ability to locate the platform in the Morris Water Maze paradigm, whereas the performance of aged rats maintained on a diet with NT-020 did not differ significantly from that of young rats (Acosta et al. 2010). This suggests that NT-020 may have rescued learning and memory in the aged rats. Furthermore, when the brains were collected and subjected to immunostaining, it was observed that aged rats on an NT-020-supplemented diet had a greater number of dividing cells in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, as determined by cell cycle marker Ki67 compared to the aged control. Additionally, NT-020-treated aged rats showed decreased activated microglia as indicated by fewer cells that expressed major histocompatibility complex II (MHCII) (by OX6 staining) in the DG than aged controls. Finally, NT-020-treated aged rats also showed
increased proliferation of neural progenitors in the SVZ, as indicated by increased doublecortin+ (DCX) staining compared to aged controls. In a double-blind, placebo-controlled trial with older adults 65-85 years of age, those taking the NT-020 supplement improved on two measures of processing speed within the two-month test period, but did not show significant improvement on other cognitive performance tests. These results collectively suggest that NT-020 exerts therapeutic effects by reversing inflammation and increasing stem cell proliferation in the neurogenic niches. While some of the therapeutic mechanisms of action for NT-020 have been defined, a complete profile of circulating factors being affected by NT-020 supplementation has yet to be elucidated.

The goal of the current study was to generate a complete list of factors from the systemic milieu that could be altered as a consequence of aging and rescued by NT-020 supplementation. We used bottom-up, discovery-based proteomics to create a profile for the entire proteome for serum derived from aged rats given NT-020 or a normal diet and young rats given NT-020 or a normal diet. Our data suggest that there are age-related molecular changes that can be rescued by NT-020 supplementation.

We designed a discovery-, mass spectrometry-based proteomics study to generate a profile of the entire rat proteome for serum derived from young (3-6 months) control, old (20-22 months) control, and old male Fisher 344 rats given a diet supplemented with NT-020. We identified some proteins that were differentially expressed between the old control and old diet groups, specifically proteins that are involved in aging and inflammatory pathways such as autophagy and complement pathways. While the findings suggest that NT-020 is exerting anti-aging effects, depletion of albumin, the most highly abundant protein in serum, presented a methodological challenge in the study. It is likely that, in spite of depletion steps undertaken to remove high abundance protein from the samples, much of the albumin and other high abundance proteins remained and may have prevented other proteins from detected.
Chapter 1 – Introduction

Aging

Aging is associated with a progressive decline in function of all cells, tissues, and organ systems throughout the body and, along with this decline in function, is an observed decrease in regenerative capacity of stem cell niches. Aging is also the primary risk factor for developing degenerative disease in both the periphery and the central nervous system (CNS). Some chronic conditions that disproportionately affect aged populations include cardiovascular disease, cancer, osteoarthritis, diabetes mellitus, Parkinson’s disease (PD), and Alzheimer’s disease (AD) (Jaul and Barron 2017, Franceschi et al. 2018, MacNee, Rabinovich and Choudhury 2014).

Figure 1. Prevalence of disease by age. Age is a significant risk factor for a number of chronic diseases. Prevalence rates of some age-related conditions are shown. Data sourced from (Harris 2013), (Alzheimer’s Association 2019), (American Heart Association 2019), (Virani et al. 2020).
According to the Centers for Disease Control and Prevention, with AD alone, age is a significant risk factor for the development of the disease and symptoms can first appear at the age of 60. As of 2014, approximately 5 million Americans were living with AD and that number is expected to triple to approximately 14 million people by the year 2060 (Centers for Disease Control and Prevention 2019). According to the Alzheimer’s Association, the greatest risk factors for developing late onset AD are: old age, carrying the e4 form of the APOE gene, and having a family history of AD. However, of these three risk factors, age is the greatest of them all, with the majority of those affected being age 65 or older. The risk for developing AD increases exponentially with age. Of the 5.8 million Americans living with AD in 2019, an estimated 5.6 million are age 65 and older. An estimated 81 percent of that 5.8 million are age 75 or older (Alzheimer’s Association 2019).

While the exact biological mechanisms driving the aging process are unknown, there are several theories that relate to damage or error, and some of these include: 1.) Programmed longevity, which states that aging is a result of specific genes selectively being switched off or on, 2.) the endocrine theory, which maintains that our hormones drive our biological clocks and this determines the rate at which we age, 3.) the immunological theory that states the immune system is programmed to decline as we age which leads to increased susceptibility to infectious diseases and being immune compromised, 4.) the “wear and tear” theory that states our cells and tissues have vital parts that simply “wear out” over time, 5.) the rate of living theory which postulates that any organism’s lifespan is determined by rate of oxygen basal metabolism, 6.) the free radicals theory that is centered around evidence that free radicals called reactive oxygen species (ROS) are able to damage cells and eventually tissues and organs (Harman 1956), 7.) the cross-linking theory that proposes there are cross-linked proteins that accumulate and damage cells and tissues over time, and 8.) the somatic DNA damage theory which states
that so much DNA damage can occur over time, and more quickly than they can be repaired, that the negative effects are cumulative and may result in mutations with age (Jin 2010).

Additionally, there is the Hayflick limit theory of aging, which was originally proposed by Dr. Leonard Hayflick in 1961. He observed that cells in culture are able to divide approximately fifty times, after which, they become senescent (Shay and Wright 2000). Another popular theory of aging is the telomere theory, which arose because telomeres have been shown to shorten with each cell division and, when telomeres become too short, cells become senescent. Some cells use the enzyme telomerase to repair telomere ends and restore length, but this function is usually limited to reproductive cells. Experiments have shown that telomerase alone cannot sufficiently restore telomere length after extensive cell division to prevent senescence (Jin 2010). A study in the central nervous system (CNS) has also shown that telomerase-deficient mice had decreased hippocampal neurogenesis and decreased neuronal differentiation (Ferron et al. 2009, Jin 2010).

The physiological processes that are thought to contribute to aging in the CNS in particular include increased oxidative stress (Zuo et al. 2019, Marosi et al. 2012), microglial activation (Angelova and Brown 2019, Wang et al. 2019), increased proinflammatory factors (Milan-Mattos et al. 2019, Tsui, Richards and Davis 2018), and decreased blood-brain barrier (BBB) integrity (Erdo and Krajcsi 2019, Bartels et al. 2009), which have been shown to result in decreased global stem cell proliferation (Wagner et al. 2019, Ahmed et al. 2017) and blunted neuroplasticity in the CNS (Acosta et al. 2010, Apple, Solano-Fonseca and Kokovay 2017).

While there are many biological theories underlying aging, the exact mechanisms are unclear. However, there is sufficient evidence to suggest that aging is complex, with more than one process occurring simultaneously, and that many of these occur at the cellular level. What is clear is that there is an overall decrease in regenerative capacity with aging.
Cell Autonomous and Cell Non-Autonomous Changes: Effects on the Stem Cell Niche

This decline in stem cell niche function is of particular significance because it results in overall decreased regenerative capacity. A number of plausible explanations, including those listed above, have been put forth in the literature to describe increased cellular senescence with age. These changes in physiology with age are often referred to as cell autonomous changes and cell non-autonomous changes. Examples of cell autonomous changes include decreased expression of tumor suppressor genes including p16\(^{\text{INK4A}}\), p53, ARF, and p21, as well as alterations to the Wnt/\(\beta\)-catenin and other canonical pathways. Cell non-autonomous changes are defined precisely how the term sounds in that they are the result of multidirectional communication between different cells and tissue or organ systems (Medkour, Svistkova and Titorenko 2016). Some cell non-autonomous changes discussed in the literature include increased CCL11 (eotaxin) which negatively impacts neurogenesis, increased presence of the pro-aging factor beta2-microglobulin (B2M), decreased brain-derived neurotrophic factor (BDNF) which is associated with age-related decreases in the volume of the hippocampus and neurogenesis (Erickson et al. 2010), decreased nerve growth factor (NGF) which is associated with decline in cognitive function (Terry, Kutiyanawalla and Pillai 2011), and decreased presence of the anti-aging growth differentiation factor 11 (GDF-11) (Katsimpardi et al. 2014, Smith et al. 2015, Villeda et al. 2011, Villeda et al. 2014). Furthermore, the neurotrophic factors BDNF and NGF have been found to be integral to the release of the neurotransmitters dopamine (DA) and glutamate (Glu) in the hippocampus and age-related loss of this function could have further implications for learning and memory in older adults (Paredes, Granholm and Bickford 2007).

Some important early research on cell non-autonomous changes has come from parabiosis studies.
Parabiosis Studies

Parabiosis studies have laid the foundation for studying the aging systemic milieu. Parabiosis entails the surgical union of the circulatory systems of two animals. Isochronic pairs consist of two animals from the same age group (young with young or old with old). Heterochronic pairs consist of a young animal and an old animal (Figure 2a). In one such study, three pairs were created by surgical parabiosis of C57BL/6 mice: young-young isochronic, old-old isochronic, and young-old heterochronic in order to study the impact of the systemic milieu on the central nervous system (CNS) (Villeda et al. 2011). There were a greater number of doublecortin (DCX)-positive stained neural cells in the dentate gyrus (DG) of the hippocampus of old mice from heterochronic pairs than old isochronic mice. In contrast, there were significantly fewer DCX^+ cells in the DG of the hippocampus of young heterochronic mice than young isochronic mice. Similarly, 5-bromo-2'-deoxyuridine (BrdU) staining of the DG of the hippocampus showed that old heterochronic mice had a greater number of BrdU^+ cells in the DG than old isochronic mice. Again, young heterochronic mice had fewer BrdU^+ cells in the DG than young isochronic mice. The results of the staining show an increase in cell proliferation and an increase in new neurons in the DG of the hippocampus of old heterochronic mice and a decrease in both cell proliferation and neurogenesis in young heterochronic mice. These data suggest that there are circulating factors in young blood that had a beneficial effect on the CNS of old mice and that there are deleterious factors in old blood that have a negative effect on the CNS of young mice (Villeda et al. 2011).

In the same study, young C57BL/6 mice were injected with either young or old plasma and subjected to the contextual fear conditioning and radial arm water maze (RAWM) behavioral paradigms. Contextual fear conditioning is a behavioral paradigm during which the animal is conditioned to associate a certain environmental context, which is the chamber, with an aversive stimulus, which is a mild foot shock, on the first day. One the second day, the
animal is put into the chamber and should exhibit freezing behavior, which is a sign of fear and anticipation of an associated foot shock. Absence of freezing behavior indicates that the animal may have impaired learning and memory. Young animals injected with old plasma displayed significantly less freezing behavior than young animals injected with young plasma, suggesting that factors, such as pro-inflammatory cytokines and chemokines, in the old plasma may have impacted learning and memory (Villeda et al. 2011).

The RAWM is a behavioral paradigm that assesses spatial learning and memory. In this test, there are multiple arms (six in this study) submerged in a pool of water, with one arm being designated as the “goal arm” with an escape platform for the animal to swim onto. Mice were trained for 3 h with 15 trials with the platform being alternated between being hidden or exposed. Swimming to an arm other than the goal arm was counted as an error. Mice injected with old plasma made significantly more errors than mice injected with young plasma in the RAWM test. This suggests that old plasma contains factors that were deleterious to spatial learning and memory (Villeda et al. 2011).

Additionally, Villeda and colleagues used proteomics to compare plasma from young and old mice to plasma from human patients in order to identify possible circulating pro-aging factors. Factors common in normal aging and parabiosis included: CCL2, CCL11, CCL12, CCL19, haptoglobin, and β-2 microglobulin (b2M). The authors identified CCL11 (eotaxin) as a pro-aging factor associated with decreased neurogenesis. To examine the effect of CCL11 on the brain, young mice were stereotaxically injected with CCL11 in the DG. A decreased number of DCX+ cells were observed in the DG compared with the contralateral side where only a vehicle control was injected. To assess the impact of CCL11 on behavior, young mice received intraperitoneal (i.p.) injections of recombinant CCL11 or vehicle control and were subjected to fear conditioning and RAWM. Animals that received CCL11 showed impaired learning and memory on both tests compared to vehicle-treated control mice. Though there are many factors
that have been identified in the literature, these data suggest that the presence of just one systemic pro-aging factor in the CNS can significantly impact neurogenesis and learning and memory (Villeda et al. 2011).

In a follow-up study by the same group, old isochronic parabionts were compared to old heterochronic parabionts to determine the beneficial effects that exposure to young blood could have on the aged CNS (Villeda et al. 2014). Immunohistochemical (IHC) staining showed an increased number of cells that expressed the genes Egr1, c-Fos, and Creb in the DG of old heterochronic compared to old isochronic mice. Additionally, Golgi staining was performed and showed increased dendritic spine density in the DG, but not CA1 region, of the hippocampus of old heterochronic mice compared to old isochronic mice.

Additionally, extracellular electrophysiology was performed to determine functional changes in the DG as a result of exposure to young blood. Isochronic parabionts had long-term potentiation that was equal to baseline levels, however heterochronic parabionts maintained LTP recordings above baseline for the whole duration, suggesting that synaptic plasticity is increased in old mice after exposure to blood from young mice (Villeda et al. 2014).

In this study, contextual fear conditioning and RAWM were carried out again to assess cognitive function, and showed the same results as before. Heterochronic old mice demonstrated increased freezing behavior in the contextual fear conditioning paradigm, suggesting increased learning and memory. In the RAWM, old heterochronic mice made fewer errors compared to isochronic old mice. These data again suggest that exposure to young blood has a positive effect on learning and memory in old mice (Villeda et al. 2014).

Based on the knowledge gleaned from their previous parabiosis studies, the group then sought to apply what they learned about the beneficial effects of young blood on old animals to Alzheimer’s disease (AD), as age is the primary risk factor for the non-familial form of AD (Middeldorp et al. 2016). Young wild-type mice were surgically joined with amyloid precursor
protein (APP) transgenic mice with the familial London and Swedish mutations, an APP mouse model which closely mimics the cognitive impairment seen in human patients with AD. As synaptic and calcium-binding proteins are downregulated in early stages of AD, staining was carried out for synaptophysin and calbindin in the DG. APP male isochronic parabionts had significantly less synaptophysin and calbindin expression in the DG, and this effect was significantly reversed in heterochronic male parabionts. The same trends were observed in female parabionts, suggesting that the beneficial effects of young blood are not sex-specific. Interestingly, high levels of amyloid-β (Aβ) remained in the hippocampus of APP heterochronic parabionts and microglial activation, as indicated by positive staining of CD68, remained. This suggests that young blood was able to restore synaptic proteins even with high levels of Aβ and inflammation.

The authors also performed genome-wide microarray analysis in order to determine genes that were differentially expressed between APP heterochronic parabionts and isochronic parabionts with the goal of identifying gene expression that was induced by exposure to young blood. The most changed were γ-secretase-activating protein (GSAP) and the synaptic lipid raft adaptor protein (PAG1), both of which are associated with the γ-secretase pathway, which cleaves APP to a form that can produce Aβ (Middeldorp et al. 2016).

Plasma injection experiments were also repeated in which middle-aged female APP mice were injected with young plasma or PBS twice a week for a month. Treatment with young plasma rescued hyper-phosphorylated ERK and returned it to the levels observed in wild-type mice, depleted synaptophysin levels were restored, and depleted calbindin levels were restored. Additionally, behavior tests were carried out in order to determine the cognitive effects of young plasma on APP mice. The Y-maze spontaneous alternation test is used to measure spatial working memory by observing an animal’s willingness to explore new environments and, in this test, there are three arms. The animal is placed in the center and allowed to freely explore the
arms. The animal should show a tendency to enter an arm that has not been recently visited as continuing to enter the same arm is indicative of cognitive impairment. APP mice treated with young plasma showed a similar number of arm entries to wild-type mice, while PBS-treated control mice performed at “chance level.” In addition to the Y-maze, mice were subjected to contextual fear conditioning and APP young plasma-injected mice showed increased freezing behavior compared to PBS controls, suggesting that young plasma had a positive effect on hippocampus-dependent learning and memory (Middeldorp et al. 2016). Together these data suggest that factors in young blood could have the potential to ameliorate disease progression in a mouse model of AD.

Collectively, data from these parabiosis studies suggest that there are circulating factors in the systemic milieu of old blood that have a deleterious, pro-aging effect and, conversely, there are circulating factors in young blood that have a beneficial, rejuvenating, and anti-aging effect. This opens up the possibility of manipulating the aged systemic milieu by introducing rejuvenating factors or somehow downregulating deleterious factors in order to restore the microenvironment to a state that is conducive to increased regeneration and repair.

**Sex Differences**

It is important to note, that while the aforementioned studies did not discuss sex differences and the current work will also not address sex differences, there are physiological differences between males and females with regard to aging and proteomes, and future work with microglia from the same animals will address some of these differences.

There are many sex differences in age-related diseases. For instance, women have a higher incidence of developing Alzheimer’s disease than men. However, women are at lower risk for stroke, though their outcome after stroke is poor compared to men (Mangold et al. 2017). As sex hormones help to drive development of the immune system, the inflammatory
response is fundamentally different between males and females and these differences are thought to contribute to disparities in disease incidence as well as differences in disease progression between males and females (van Lunzen and Altfeld 2014). In the brain, the resident immune cells, microglia, also display key differences between the sexes with regard to cell density, localization of the cells, transcription, and translation (Guneykaya et al. 2018). A previous study showed male and female mice had 1,209 differentially expressed genes in the hippocampus alone, 55 in the cortex, and 46 differentially expressed genes in both regions of the brain. In proteomic analysis with microglia from whole brain samples, 268 proteins were found to be expressed at significantly higher levels in microglia from male mice and 96 proteins were found to be expressed at significantly higher levels in microglia from female mice (Guneykaya et al. 2018).

Another study analyzed the sex differences in the neuroinflammatory responses of microglia in the hippocampi of young (3 months), adult (12 months), and old (24 months) mice (Mangold et al. 2017). Whole hippocampi were used for bioinformatic analysis of gene and protein expression. Female mice had an overall greater change in gene expression with age compared to males with age and that change was significant between young and adult and adult and old, suggesting the change was age-dependent across the lifespan. Male mice had more variability in gene expression between animals. Pathways associated with inflammation were activated in an age-dependent fashion in both males and females, but with females there was an even greater immune activation later in the lifespan. There was also significant age-dependent differential microglia-specific gene and protein expression between the sexes, with more increases in expression of microglial ligands and pro-inflammatory genes in females than males. In addition, females showed significantly greater age-dependent expression of complement protein C1q than males (Mangold et al. 2017).
Females and males clearly experience different physiological changes with age and present a different phenotype with respect to microglia in preclinical research, which also suggests that activation state may not be the only important piece of information to glean about microglia. Bioinformatics provides a unique opportunity to further elucidate some of these sex differences. Future work from our group will aim to elucidate some of these age-dependent sex differences using microglia from the animals used in the present study.

Graft Studies

In addition to parabiosis studies, studies of graft transplantation have also demonstrated that there are deleterious factors in the aged milieu, such as circulating pro-inflammatory cytokines and reactive oxygen species. These circulating factors can impact survival of the graft in the aged host. One study showed that transplantation of hippocampal embryonic neuronal cells resulted in significantly less microglial activation (shown by Ox6 and Iba1 staining) after cells were transplanted intraocularly into 17-18 month-old rats after the rats were maintained on a 2% blueberry-supplemented diet than age-matched controls. In addition, blueberry-supplemented aged rats had less astrogliosis, as determined by staining for glial fibrillary acidic protein (GFAP) than age-matched controls. Furthermore, these effects were significantly more pronounced when rats were maintained on the blueberry diet long term (8-12 weeks) as opposed to short-term (1-2 weeks) (Willis et al. 2010). Blueberry supplementation has also been shown to support the survival and growth of these grafts (Willis et al. 2008) These findings support that there are negative factors in old blood and that they can be ameliorated by dietary supplementation with antioxidants.
Nutraceuticals for Aging and Age-related Degenerative Disease

The term “nutraceutical” is a broad, umbrella term for all types of food with health or medical benefits and many are being applied to conditions affecting older adults (Vranesic-Bender 2010). Some of the conditions that disproportionately affect aging populations include hypertension, diabetes, dementia, osteoporosis, respiratory problems, cataract, and heart disease (Gupta and Prakash 2014). Most nutraceuticals are hypothesized to target free radicals or reactive oxygen species (ROS) which result in oxidative stress in aging. A beneficial effect of using a nutraceutical is thought to be the result of antioxidant activity, and some of the types of substances that are used for their antioxidant activity include flavonoids, carotenoids, vitamins, and minerals. In addition to the oxidative stress hypothesis, genetic factors termed “longevity-related genes” have also been shown to play a role in the health and lifespan of yeast, C. elegans, flies, and rodents. There is also the “hormesis” hypothesis of aging which states that protective mechanisms can be elicited, in this case by dietary supplements or nutraceutical intervention, to influence expression of genes.

Chemically, nutraceuticals may be classified in a number of ways including: isoprenoids (carotenoids, saponins, terpenes, etc.), phenolic compounds (tannins, anthocyanins, isoflavones, flavones, flavonoids, etc.), carbohydrates (ascorbic acid, oligosaccharides, nonstarch polysaccharides), fatty acids (n-3 polyunsaturated fatty acids, etc.), amino acid derivatives (folate, choline, etc.), microbes (prebiotics and probiotics), and minerals (Ca, Zn, K, etc.) (Gupta and Prakash 2014).

One study found that with aging there were increased levels of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-6 (IL-6), and IL-8 in serum from aged rats as determined by enzyme-linked immunosorbent assay (ELISA). Additionally, there was decreased expression of upstream antioxidant genes DJ-1 and NRF2 and increased expression of apoptosis-related genes p53 and Bax (mRNA) in the
thymus, as determined by quantitative real-time polymerase chain reaction (RT-PCR). Rats were either untreated (control), given ethanol and phosphate buffered saline (PBS) (vehicle control), had 10mg/kg body weight melatonin added to their diet (Aged+Mel), or had 2% turmeric added to their diet (Aged+Tum). Treatment with either the antioxidants melatonin or turmeric reversed these age-induced changes in physiology (Ismail, El-Bakry and Soliman 2018).

Another study showed that standardized turmeric extract HSS-888 was able to reduce Aβ deposition and neurofibrillary tangle-producing phosphorylated tau in a transgenic model of Alzheimer’s disease (AD) (Shytle et al. 2012). A diet supplemented with HSS-888 resulted in reduced presence of amyloid in the cerebrum of transgenic mice as determined by immunohistochemical (IHC) staining. Additionally, treatment with HSS-888 resulted in reduction of both soluble and insoluble forms of Aβ, as determined by ELISA. Furthermore, HSS-888 diet supplementation resulted in significantly decreased phosphorylated tau protein in brain homogenate, as determined by Western blot. Microglia from the animals were subjected to cytokine analysis by ELISA and it was found that mice treated with HSS-888 had significantly increased expression of anti-inflammatory cytokines IL-2 and IL-4. These data suggest that HSS-888 had therapeutic effects in transgenic AD mice by direct disease-specific mechanisms such as reducing plaque and neurofibrillary tangle burden, but also indirectly through anti-inflammatory mechanisms (Shytle et al. 2012).

Another nutritional approach to combating oxidative stress and inflammation in the periphery as well as the CNS is the ketogenic diet or supplementation with exogenous ketones. One trial showed that giving patients a mixture of medium chain triglycerides (MCTs) improved memory and attention in AD patients (Reger et al. 2004). In transgenic APP+ presenilin 1 (APP+PS1) mice, however, MCT treatment only improved motor function and not cognition on behavioral paradigms, nor did it have any effect on neurogenesis, astrocytosis, nor microglial
activation (Brownlow et al. 2013). However, MCT dietary supplementation has been shown to mediate inflammation by blocking activity of the NLRP3 inflammasome, which controls activation of caspase-1 and macrophage production of pro-inflammatory cytokines IL-1β and IL-8 (Youm et al. 2015). Moreover, the risk for developing cancer increases with age and exogenous ketone supplementation has been shown to be anti-tumorigenic in mice (Poff et al. 2014).

Polyphenolic Compounds and Diet

In addition to the compounds mentioned, polyphenolic compounds have also been shown to increase rejuvenation in aging and age-related diseases. Polyphenols are naturally occurring compounds that are plant secondary metabolites and are considered anti-aging supplements due to their ability to modulate some of the physiological hallmarks of aging including inflammation, oxidative stress, cellular senescence, and autophagy (Russo et al. 2019, Tressera-Rimbau et al. 2017). Polyphenol-rich foods include berries, green tea, fruit juice, coffee, nuts, and cocoa (Rajaram, Jones and Lee 2019).

In a recent study, young and old male Wistar rats were given grape juice orally for 28 days (10 μL/g body weight). Ferric reducing ability of plasma (FRAP), ROS, plasma membrane redox system (PMRS), glutathione (GSH), osmotic fragility, and decrease in lipid peroxidation by malondialdehyde (MDA) levels were measured. There was a significant increase in the antioxidants FRAP, PMRS, and GSH and a significant decrease in ROS and MDA in treated rats versus controls, suggesting that grape juice was able to quell oxidative stress signaling (Kumar, Bhoumik and Rizvi 2019).

Another study examined the anti-aging and microbiome effects of life-long consumption of polyphenols from lemon (LPP) when administered to a mouse model of aging, the senescence-accelerated mouse prone 1 (SAMP1) and a resistant strain, senescence-
accelerated resistant mouse 1 (SAMR1) (Shimizu et al. 2019). The SAMP1 mouse model shows early signs of aging including senile amyloidosis, impaired immunity, and decreased motor function and the SAMR1 model is often used as an age-matched control. LPP (0.1%) was added to tap water and mice were allowed ad libitum access. Grading of age-related changes was accomplished by calculating scores for skin and hair condition (glossiness, coarseness, hair loss), ulcers, condition of the eyes (cataract, lesions, clarity of cornea, ulcers), and skeleton (spinal curvature) every month up to 88 weeks of age. Mice were subjected to the behavioral paradigms novel object recognition test (ORT) and object location test (OLT) at various timepoints throughout the lifespan in order to assess spatial cognition. Normal mice should spend more time exploring a novel object than a previously encountered object on the ORT and normally functioning mice should be able to recognize when an object has been moved to a new location in the OLT. Locomotor activity was measured by movement inside the ORT box.

Analysis of the microbiome was carried out by isolating bacterial DNA from feces and conducting PCR. The LPP-treated SAMP1 group was significantly lower in age-related scores for periophthalmic lesions, hair coarseness, and hair loss than the plain water control group. LPP-treated SAMP1 mice also showed significantly greater motor activity compared to the plain water control group, although it did not reach the levels of the SAMR1 age-matched control. LPP treatment also significantly improved long-term object memory and spatial recognition, as measured by ORT and OLT, in SAMP1 mice. The gut microbe *Lactobacillus* increases with age in human subjects and, interestingly, the *Lactobacillus* in SAMP1 LPP-treated mice at 70 weeks of age, was significantly lower than those in the plain water control group. Collectively, the results of the study suggest that lifelong consumption of LPP had anti-aging effects on overall health and on the intestinal microbiome in SAMP1 mice.

As previously mentioned, there is a preponderance of evidence that vulnerability to oxidative stress is one major factor contributing to age-related decline, particularly in the CNS,
and research on antioxidant-rich diets show a number of therapeutic effects. In a study where 6-month-old rats were given a diet supplemented with strawberry extract (SE 9.5 gm/kg dried aqueous extract), spinach extract (SPN 6.4 gm/kg dried aqueous extract), or vitamin E (500 IU/kg) for two months, SPN had the greatest effect on cognitive performance as assessed by the Morris water maze paradigm, but vitamin E had the greatest effect on mitigating oxidative stress as assessed by 2′7′-dechlorofluorescein diacetate analysis. This suggests that phytochemicals in such compounds may be beneficial for decreasing oxidative stress (Joseph et al. 1998). Another study by our group demonstrated that a diet supplemented with antioxidant-rich blueberry and spirulina extract induced transient activation of microglia soon after 6-OHDA injection into the dorsal striatum of normal rats, but the number of activated microglia significantly decreased in diet-treated rats one month after lesion compared to controls. The blueberry-spirulina diet group also had greater recovery of dopamine neurons, as evidenced by tyrosine hydroxylase staining, in the striatum and the globus pallidus, than the control group given a standard diet (Stromberg et al. 2005).

Transplantation of therapeutic cells or neural tissue have received attention as potential therapies for neurodegenerative diseases, however the age of the host has a significant impact on graft success and survival, with a more aged microenvironment having negative impacts on transplants. A study using young and middle-aged female Fisher 344 rats as donors receiving intraocular grafts from fetal hippocampi were randomly assigned to receive a standard NIH-31 chow or chow supplemented with 2 % blueberry, which are rich in antioxidants. Animals were subdivided again to receive diet either short-term (1-2 weeks) or long-term (8-12 weeks). The results revealed that compared to young animals, old control animals had significantly less graft growth. However, blueberry-treated grafts in old rats had grafts that grew just as well as, and sometimes even better than, young control rats. In addition, blueberry-treated rats also had improved graft organization that resembled that of young animals compared to old control rats.
Furthermore, the final size of the grafts was similar between blueberry-treated old rats and young rats (Willis et al. 2008).

Together, these studies provide evidence that flavonoids and polyphenolic compounds are able to reverse some of the deleterious processes that occur with aging such as increased oxidative stress, decreased stem cell proliferation, and increased inflammation.

**NT-020**

NT-020 (Nutrastem™, Natura Therapeutics, Inc.) is a nutraceutical compound composed of a proprietary blend of polyphenols blueberry and green tea, the amino acid carnosine, and vitamin D3 that was developed at the Center of Excellence for Aging and Brain Repair at the University of South Florida. The group had found that blueberry, green tea, catechin, carnosine, and vitamin D3 synergistically induced the proliferation of human bone marrow cells, human CD34+, and human CD133+ cells in a dose-dependent manner compared to human granulocyte-macrophage colony-stimulating factor (hGM-CSF). Furthermore, the combination of the ingredients blueberry, green tea, vitamin D3, and carnosine produced greater proliferation in CD34+ cells and CD133+ cells than each of the individual components alone. This demonstrates that this combination, now NT-020, had a synergistic and additive effect on cell proliferation (Bickford et al. 2006).
Figure 2. **NT-020 promotes CD34+ cell proliferation.** Compounds in NT-020 promote proliferation of CD34+ cells in a synergistic manner and its effect is greater than its individual ingredients. Image from Bickford et al. (2006).

Treatment with NT-020 was also found to exert therapeutic and neurogenic effects in a rat model of ischemic stroke. Adult male Sprague-Dawley rats were randomly assigned to be treated with NT-020 or a vehicle control by daily oral gavage for two weeks prior to stroke induction. On day 14, ischemic stroke was modeled by middle cerebral artery occlusion (MCAo). Rats were subjected to behavioral tests, including the Bederson test and the elevated body swing test (EBST), before and after MCAo surgery. The Bederson test is commonly used to
measure outcome after stroke. Parameters include 1) observed spontaneous ipsilateral circling (graded 0-3, 0 = no circling, 3 = continuous circling), 2) contralateral hindlimb retraction which measures the rodent’s ability to replace the hindlimb after it has been displaced (graded 0 (immediate replacement) to 3 (replacement)), 3) beam walking (graded from 0 (rat walks across a 2.4 cm-wide, 80 cm-long beam without an issue) to 3 (rat is unable to stay on the beam for ten seconds (s)), 4) bilateral forepaw grasp which measures the ability to hold onto a 2 mm-diameter steel rod (graded 0 (for normal forepaw grasping) to 3 (for unable to grasp)). When the tests are completed, the scores from all four tests are added and averaged to determine a neurological deficit score. At 14 days after stroke surgery, both the NT-020 group and the vehicle control group showed neurological deficits, however the NT-020 group had significantly fewer neurological deficits, as determined by the Bederson test.

The EBST involves lifting the rodent by the base of the tail until the nose is about 2 inches about the surface. The direction that the animal swings (right or left) is recorded. After each individual swing, the animal is set down to rest before re-testing, and the test is repeated twenty times. Animals functioning normally will have a 50% swing bias for the right or left, but MCAo stroke model animals tend to exhibit a 75% swing bias for a particular side. Similar to the results of the Bederson test, both the NT-020 group and the vehicle control displayed motor deficits, as determined by EBST, following stroke surgery, however NT-020-treated rats had significantly fewer deficits than the vehicle-treated rats (Yasuhara et al. 2008).

Additionally, glial fibrillary acidic protein (GFAP) staining was used to visualize glial scarring, and it was found that with NT-020 treatment there was significantly less glial scarring. 5-bromo-2’deoxyuridine (BrdU) labeling of brain tissue revealed that NT-020 increased the number of proliferating cells in the subventricular zone (SVZ) and the striatum compared to the vehicle-treated control group. The results also suggest that NT-020 treatment enhanced differentiation of nascent cells to a neuronal phenotype in the striatum, as determined by
increased double-positive staining of cells for BrdU and doublecortin (DCX) compared to vehicle-treated controls. Furthermore, NT-020 treatment did not enhance differentiation of nascent cells to a glial lineage, as exhibited by less double staining for BrdU and GFAP compared to vehicle-treated controls. Altogether the results suggest neuroprotective effects of NT-020 diet supplementation for stroke prophylactically (Yasuhara et al. 2008).

With aging there is a significant decrease in stem cell regenerative capacity and cells are more vulnerable to oxidative stress. Blue-green algae is among some of the flavonoids researched for its ability to aid in endogenous stem cell repair. As NT-020 was previously shown to promote stem cell proliferation, a study was designed to ascertain whether blue-green algae (Aphanisomenon flosaquae (AFA)) and NT-020 could synergistically promote cell proliferation. Human bone marrow cells or human CD34+ cells were cultured with NT-020, different concentrations of AFA, or both NT-020 and AFA (500 ng/ml), and MTT assays were carried out to measure cell proliferation. The results showed that AFA and NT-020 significantly increased cell proliferation in both human bone marrow cells and human CD34+ in an additive manner (Shytle et al. 2010).

In a follow-up study, human neural progenitor cells, human bone marrow cells, and human CD34+ cells were cultured with various doses of spirulina or NT-020, or a combination of the two. Both spirulina and NT-020 increased proliferation of human bone marrow cells, as determined by MTT assay. However, the combination of spirulina and NT-020 significantly increased proliferation more than either spirulina or NT-020 alone. The same effect was observed with CD34+ cells. However, surprisingly, for NPCs spirulina and NT-020 alone increased proliferation significantly, however the combination decreased proliferation to levels below the control (media alone) (Bachstetter et al. 2010).

NT-020 was also shown to enhance cognitive function, increase proliferation of neural progenitor cells, and decrease inflammation in aged rats. Young (3-month-old) or old (20-month-
old) rats were either treated with NT-020 or water (control) by oral gavage for four weeks. Spatial memory was assessed by MWM, which was measured over 5 days of training with four trials per day. Old rats treated with NT-020 performed better on the MWM test (Acosta et al. 2010).

Figure 3. Aged rats on NT-020 show improved cognitive function. Old rats on NT-020 made fewer errors in locating the platform on the Morris Water Maze (MWM) than age-matched controls (Acosta et al. 2010).

Staining with the cell cycle marker Ki67 revealed a significant age-related decrease in cell proliferation in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus, however in old rats treated with NT-020 there was a significant increase in cell proliferation compared to age-matched controls. Similarly, staining with the marker for immature neurons, doublecortin (DCX), significantly decreased with age. Treatment with NT-020 showed a
significant increase in DCX in the DG of the hippocampus in old rats compared to age-matched controls (Acosta et al. 2010).

Ki67 staining was also performed to visualize cell proliferation in the subventricular zone (SVZ), and like the SGZ of the DG, there was an overall age-related decrease in Ki67+ cells compared to young rats. However, with NT-020 treatment there was an increase in cell proliferation in old rats, as determined by Ki67 staining, compared to age-matched controls.

**Figure 4. NT-020 increased neurogenesis in the hippocampus of old rats.** Ki67, a marker of cell proliferation and growth, was detected in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. There was blunted Ki67 in the SGZ in old rats, however this was reversed with NT-020. Likewise, there was blunted positive staining for doublecortin (DCX), a marker for immature neurons, in the SGZ with age, however this was reversed with NT-020 (Acosta et al. 2010).

Aging is associated with an increase in inflammation, both in the periphery and the CNS. The predominant immune cells of the brain are microglia, which express major histocompatibility complex II (MHCII) when in the activated state. In order to determine whether NT-020 treatment
decreased the number of activated microglia, OX6 staining was carried out to reveal the number of MHCII-expressing cells in the DG. There was an age-related increase in OX6+ cells in the DG compared to young rats, however with NT-020 treatment there was a decrease in the number of OX6+ cells compared to age-matched controls. The evidence in the study demonstrated that NT-020 treatment increased neurogenesis, decreased microglial activation, and enhanced cognitive function in aged rats (Acosta et al. 2010).

In a double-blind, placebo-controlled clinical trial by our group and a partnering center, one hundred and five older adults (aged 65-85 years) were randomized a pill-based form of 900 mg of blueberry, green tea, and carnosine plus 200 U of vitamin D3 (NT-020) and 40 mg Biovin (grape extract) to assess the impact of NT-020 on cognitive function. Participants were administered a battery of cognitive tests to establish baseline scores, and then they were given NT-020 pills or placebo to take orally for two months total, with a blood draw at the one-month point. Participants also kept a pill and symptom diary to track when they took the pill and any adverse events throughout the course of the study. After the two-month period, participants returned to have cognitive measures administered again. The cognitive measures assessed episodic memory, processing speed, verbal ability, working memory, executive functioning, and complex speed.

The NT-020 group displayed significantly better performance than the placebo group on the Identical Pictures Test. There was also a trend towards significance for the NT-020 group versus the placebo group on the Number Comparison task. Interestingly, both of these tests are measures of processing speed. As the participants enrolled in the study did not have any cognitive impairment, the absence of robust improvement across all domains may not be surprising. Additionally, as processing speed is a domain that often significantly changes with age, it may be expected that this domain would be amenable to change with a therapeutic compound, such as NT-020 (Small et al. 2014, Lindenberger, Mayr and Kliegl 1993, Salthouse
The results of the study show that NT-020 was well-tolerated and facilitated improvement in processing speed, suggesting that NT-020 is a promising potential intervention for improving cognitive function in older adults (Small et al. 2014).

As previously discussed, aging is associated with decreased stem cell proliferation. This is attributed, in part, to dysregulated signaling in stem cell niches. In particular, Wnt signaling has been identified as neurogenic and declines with age. As NT-020 had previously been shown to enhance stem cell proliferation in neurogenic niches, a study was carried out to determine whether NT-020 could also enhance Wnt signaling in aged neurogenic niches. Young (3-month-old) or old (20-month-old) male Fisher 344 rats were randomly assigned to be maintained on standard NIH-31 chow or to be fed a chow supplemented with NT-020 (135 mg/kg body weight) for one month. Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to detect relative gene expression of WNT signaling targets. Several WNT target genes changed with age. Genes associated with cell cycle including Cyclin D1 (-1.6 fold), C-Myc (-2.1 fold), and AHR (-2.12) decreased. The growth factors glial cell line-derived neurotrophic factor (GDNF, -2.7 fold) and insulin-like growth factor (IGF, -3.6 fold) decreased. In old rats treated with NT-020, the growth factors GDNF (2.23 fold) and fibroblast growth factor 4 (FGF4, 1.89 fold) increased. GDNF and FGF4 are factors which promote the survival of neural progenitor cells (Flowers et al. 2015, Flowers et al. 2016). Immunohistochemical (IHC) staining was used to identify Wnt downstream targets Nrf2 or β-catenin colocalized with newborn neurons (doublecortin (DCX) NeuN, astrocytes (glial fibrillary acidic protein (GFAP), or microglia (Iba-1) in the dentate gyrus (DG) of the hippocampus or the subventricular zone (SVZ). With NT-020 treatment, Nrf2 colocalized with all cell types in both neurogenic niches, the DG and the SVZ, compared to old controls. Similar to the results with Nrf2, compared to control, rats on the NT-020 diet had a higher percentage of β-catenin co-localization with all cell types. Both the
Nrf2 and β-catenin IHC results support increased overall Wnt activity in old rats as a consequence of dietary supplementation with NT-020 (Flowers et al. 2015, Flowers et al. 2016).

As previously mentioned, chronic inflammation is considered a hallmark of aging. In addition to the aforementioned measures, the expression of 84 cytokine and chemokine genes in the hippocampus was measured using an RT-PCR cytokine array. The protein concentrations of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin -1beta (IL-1β) were also measured using an enzyme-linked immunosorbent assay (ELISA). The results of the cytokine array revealed that there was a significant increase in expression of the anti-inflammatory cytokines IL24, IL4, and IL10 in the NT-020 old diet group versus controls. Additionally, with NT-020 there was increased expression of CXC3CL1 (fraktalkine), LIF, and GDNF, which suggests that there was pro-regenerative, alternative M2 activation of microglia. Furthermore, there was decreased expression of the pro-inflammatory cytokines IL-1α and IL18 as well as decreased expression of the T cell chemokines CCL19 and CCL2 with NT-020 diet. The results of the ELISA assay revealed that the protein levels of TNF-α and IL-1β were significantly increased in the hippocampus with age, however this was reversed with NT-020 diet. Taken together, the results of the study indicate that NT-020 is able to target signaling pathways that are pro-neurogenic and modulate inflammation to improve outcomes for aging (Flowers et al. 2015, Flowers et al. 2016).

As previously discussed, it has been suggested that one of the reasons for the decline in function of stem cells niches with age is due to cell non-autonomous changes. That is, there is evidence that there are negative factors in aged systemic milieu that impact stem cell niches. Likewise, there is a lack of rejuvenating factors in aged blood that exist in young blood, and this absence of rejuvenating factors may also contribute to age-related decline in stem cell proliferation. To that end, a study was conducted to compare the effects of young blood, old blood, and old blood from rats maintained on a diet supplemented with NT-020 on stem cell
proliferation. Young (aged 3-6 months) and old (aged 20-22 months) rats were randomly assigned to be fed a standard NIH-31 chow or NIH-31 chow supplemented with NT-020 with Biovin (135 mg/kg/day body weight). Rats were sacrificed after being maintained on the diet for 28 days. Blood was collected by cardiac puncture and serum was isolated.

Rat hippocampal neural progenitors (NPCs) and rat mesenchymal stem cells (MSCs) were cultured with serum from young control, old control, young-NT-020, or old-NT-020 serum. 5-bromo-2’deoxyuridine (BrdU) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out to measure cell proliferation. With the MTT assay, NPC results revealed a significant decrease in proliferation with age. However, interestingly, NT-020 not only significantly increased proliferation of NPCs compared to old controls, but proliferation reached levels greater than even young rats. With the BrdU assay, NPCs showed an age-related decline in proliferation that was significantly reversed when NPCs were cultured with serum from old rats maintained on an NT-020 diet. The results of the MTT assay with MSCs showed an age-related decrease in proliferation, however the proliferation levels of MSCs cultured with serum from young NT-020 rats and old NT-020 rats were comparable. The BrdU assay results for MSCs showed that there was an age-related decrease in proliferation, however MSCs treated with serum from old rats maintained on an NT-020 diet had significantly greater proliferation than age-matched controls. There were no significant changes in serum metabolite levels with NT-020 for CCL11 (eotaxin), CCL1 (MCP-1), BDNF, or CRP. Though there were no specific mechanistic insights in the study, the data revealed a beneficial effect of NT-020 on stem cell proliferation and the ability of NT-020 to reverse age-related dampening of proliferation (Bickford et al. 2015).
Figure 5. **NT-020 rescues negative effects of old blood on NPCs and MSCs in vitro.** The results of MTT assays and BrdU assays show that exposure to old serum had a deleterious effect on cell proliferation for both neural progenitor cells (NPCs) and mesenchymal stem cells (MSCs), but treatment with serum from rats treated with NT-020 resulted in significantly improved cell proliferation (Bickford et al. 2015).

Thus far, the data obtained for NT-020 demonstrate that this polyphenol-rich nutraceutical is able to increase stem cell proliferation, increase neurogenesis, decrease oxidative stress, decrease inflammation, and improve cognitive function for aging and age-related conditions such as stroke. While the data are indicative of therapeutic effects exerted by NT-020, the mechanisms of action require further study. Previous studies showed that NT-020 is able to change the aged systemic milieu to one that supports stem cell proliferation, but specific proteins and genes being affected within the aged milieu with NT-020 dietary supplementation had not yet been fully elucidated.
Summary of Approaches and Project Aim

With aging, there is a functional decline of all systemic cells, including stem cell niches, both in the periphery and in the CNS. This decline in stem cell proliferation results in decreased regenerative capacity. There are a number of mechanisms that underly this decreased cellular regeneration with age, including cell autonomous changes as well as cell non-autonomous changes. Parabiotic studies in which the circulatory systems of old mice were connected with the circulatory systems of young mice revealed that young blood conferred benefits to old animals, while old blood conferred deleterious effects to young animals in terms of neurogenesis. Additionally, injection of young animals with old plasma resulted in poor performance on cognitive behavioral paradigms. Thus, circulating factors in the blood can impact the CNS. This is consistent with evidence that there is increased blood-brain barrier (BBB) permeability with aging and, therefore, increased vulnerability of the CNS to molecules and other environmental factors from the periphery.

A study conducted in our laboratory showed that when serum from old rats was used in stem cell culture, the aged serum had a negative impact on stem cell proliferation. However, when cells were cultured with serum from rats treated with NT-020, there was increased proliferation. The beneficial properties of young blood and the deleterious properties of old blood have not yet been fully elucidated, however there is such a preponderance of evidence that oxidative stress and chronic inflammation contribute to aging that the term “inflammaging” has been coined. This is particularly salient in the CNS, where inflammation is known to contribute to a litany of age-related neurodegenerative diseases.

Our group has focused on age-related pathologies as well as non-pathological or “healthy” aging (aging without the occurrence of disease). Our laboratory has studied various nutraceutical compounds. NT-020, a proprietary blend of blueberry, green tea, carnosine, and
vitamin D3, has been studied in particular for its ability to promote endogenous neurogenesis, decrease inflammation, and contribute to recovery from age-related conditions such as stroke.

In a previous study, it was shown that neural progenitor cells (NPCs) and mesenchymal stem cells (MSCs) cultured with serum from old rat serum had decreased proliferation compared to those cultured with young rat serum. However, when NPCs or MSCs were cultured with serum from old rats treated with NT-020, proliferation was shown to increase compared to age-matched controls. However, there were no significant changes in the expression of the factors CCL11, CCL2, BDNF, nor CRP with NT-020 treatment (Bickford et al. 2015).

Thus, while the data have shown a lot of potential for NT-020 as a therapeutic compound, further study is needed to elucidate the specific factors underlying the increased proliferation and other beneficial effects observed with NT-020 dietary supplementation. Therefore, we designed a bottom-up, discovery-based, and mass-spectrometry-based proteomics study to analyze the full serum proteome of aged rats treated with NT-020 versus young control, young NT-020-treated, and age-matched control rats.

We predict that proteomic analysis will reveal that NT-020 treatment in aged rats is associated with decreased inflammatory factors, increased anti-inflammatory factors, decreased pro-aging factors, increased factors associated with stem cell proliferation such as growth factors, and increases in anti-aging factors (such as factors that regulate oxidative stress).

Findings from this study could provide key mechanistic insights into, not only which pathways are affected by NT-020 treatment, but may also serve to identify other potential therapeutic targets for future studies of aging and age-related pathologies that affect the CNS or the periphery. We anticipate that NT-020 will be associated with downregulation of inflammatory and pro-aging factors such as factors promoting oxidative stress, or that inhibit stem cell proliferation.
Chapter 2 – Proteomics

Mass Spectrometry and Proteomics

Mass-spectrometry (MS)-based proteomics is a method that allows for the complete and unbiased characterization of proteins on a large scale. This method also allows for the study of expression changes, localization, interaction, domain structure, activity, and post-translational modifications (PTMs). Unlike other methods, MS does not rely on antibodies to bind to proteins in order to detect changes in expression and it achieves greater depth of analysis. This technique also allows for the mass quantification of results and comparison between treatment groups (Han, Aslanian and Yates 2008, Savaryn, Toby and Kelleher 2016).

MS creates ions from analyte (sample) molecules and then separates these ions based on charge and mass. MS measures the mass-to-charge ratio (m/z) of ions in the gas phase. Mass spectrometers have three basic parts: an instrument that converts the substance (the analyte) into gas-phase ions, such as a liquid chromatographer, a mass analyzer that separates the analytes based on the m/z ratio, and a detector that records the number of ions for each m/z value (Han et al. 2008, Savaryn et al. 2016).

The mass analyzer is the most important component in MS and there are many different types of mass analyzers, however one of the newest types being widely used is the Orbitrap, which was invented in 1999 and just started being used for proteomics in 2005 (Hu et al. 2005). The LTQ-Orbitrap (Thermo Fisher Scientific) is so named because ions are trapped within it and orbit around an electrode in an oscillatory motion, producing a frequency that corresponds to their respective m/z values. This induces a current in outer electrodes that is transformed into mass spectra (Han et al. 2008, Pan et al. 2010).
Fragmentation is necessary for analysis of proteins. Tandem mass spectrometry (MS/MS) is a sequencing technique for proteins and peptides. With tandem mass spectrometry (MS/MS), two mass analyzers from the same instrument generate fragments from an analyte in order to give structural information about it. Collision-induced dissociation (CID) is the most commonly used of these MS/MS techniques. CID involves internal heating of gas-phase peptide ions by collisions, generating heat and breaking apart peptide backbones. Another fragmentation technique that is newer is electron-capture dissociation (ECD) which involves fragmenting peptide backbones by capturing thermal electrons (Han et al. 2008, Cappadona et al. 2012, Pan et al. 2010).

Protein identification via M/S can be either top-down, which can be carried out by analyzing whole proteins, or by bottom-up proteomics, which involves the analysis of peptides fragmented either enzymatically or chemically. Bottom-up proteomics tends to be used with more complex samples.

Top-down proteomics involves ionization of intact proteins without the need for prior digestion, and high-resolution measurement of their mass. Afterward, they are fragmented inside the mass spectrometer. This approach, however, is difficult for large proteins (>5kDa). In this event, a “middle-down” approach can be undertaken in which there is partial digestion of large peptides. Conversely, bottom-up proteomics entails digestion of proteins into peptides before loading samples into the mass spectrometer, and the process involves the inference of the presence of specific proteins by identification of constituent peptides. A previous method used would involve pre-sorting and separating prior to protein digestion and then using peptide mass fingerprinting (PMF) or peptide separation by mass using a liquid chromatographer interfaced to a tandem mass spectrometer. Now, more commonly, the approach is to perform protein digestion in advance and then allow proteins to be sorted and separated by multi-dimensional chromatography and then tandem mass spectrometry analysis. The data collected
from tandem mass spectrometry are searched by an algorithm and compared to an existing database of proteins that is based on genomes from multiple species to identify the peptides in the sample.

Quantitative techniques allow for the comparison of control groups with treated groups, and a number of quantitative techniques use isotope labeling of proteins or peptides, such as isotope-coded affinity tags (ICAT). There are other techniques that have since been developed and another involves labeling with amino acids (usually arginine and lysine), called stable isotope labeling with amino acids in cell culture (SILAC). After the peptide backbone is cleaved with trypsin, all resulting peptides have a label with an amino acid. However, a drawback is that SILAC’s use is limited to cultured cells and it does not work well for other types of samples such as tissue. There is also enzymatic labeling, which uses H$_2^{18}$O as the peptide bond is hydrolyzed and an additional $^{18}$O can be added. The reaction is less efficient than other methods and is entirely dependent on enzymatic activity so it has not been a preferred method. Additionally, a highly sensitive mass spectrometer is required to detect enzymatic labeling. Another method of quantification is isobaric tags for relative absolute quantification (iTRAQ), which uses isotope covalent tags to quantify peptides and, like previously mentioned methods, it can label peptides after the digestion step. However, now the standard is to use label free quantification (LFQ). Normalization of samples is important for obtaining accurate LFQ and normalization features are included in software used to generate LFQ intensity values, such as MaxQuant, which was created by the Max Planck Institute of Biochemistry (Han et al. 2008, Cappadona et al. 2012, Krey et al. 2014).
Figure 6. General workflow for mass spectrometry-based proteomics. Cells or tissue are lysed and proteins are digested to peptides using a hydrolyzing protease, such as trypsin. Peptides are then fractionated before being subjected to mass spectrometry. Raw data are compared to existing protein sequence databases on different species (obtained from genome information) uploaded to software programs. The result are identified proteins that can be used in analysis. The image is credited to the Proteomics Core Facility, College of Arts and Sciences, University of South Florida, Tampa, FL, USA.

Tissue-Specific Proteomics Using Serum

Mass spectrometry proteomics using serum has many clinically relevant applications as there are many biomarkers for disease that can be detected in serum. Furthermore, obtaining a blood sample from which serum can be isolated is non-invasive and cost-effective so using this biomaterial for MS-based proteomics could be highly useful for disease diagnosis and prognosis (Del Pilar Chantada-Vazquez et al. 2020).
However, working with serum presents some technical challenges and necessary steps to address them. Both plasma and serum contain a large amount of high abundance proteins, most notably albumin, the presence of which can interfere with detection of lower abundance proteins. According to Han et al. (2008), not all digested peptides in a sample are observable and it can be especially hard to detect proteins with unanticipated modifications. Additionally, MS analysis by nature tends towards preferential sampling of peptides at high abundance over those of low abundance. Moreover, according to the Broad Institute, LFQ is also tied to abundance and results can vary depending upon instrument settings and the number of peptides in a given protein. LFQ is considered reliable only for highly abundant proteins and the results can produce a lot of missing data, particularly for lower abundance proteins (Carr). Therefore, this necessitates the addition of a depletion step in which highly abundant proteins are depleted in the samples before further processing (Lee et al. 2019).

Sample Preparation and Albumin Depletion

Over the course of the project, we tested the efficacy of a number of different high abundance protein depletion methods in order to remove high abundance proteins, such as albumin and IgG, from sera. The first depletion method we used was a homemade trichloroacetic acid (TCA) precipitation of proteins, which is a method commonly used to remove contaminants from samples. We tested 4% SDS and 8M urea to lyse and solubilize the sample and ended up choosing SDS. Ultimately, the concern with using this method for depletion was the non-specific precipitation, meaning proteins in the sample other than the high abundance proteins could be precipitated as well (Liu et al. 2014) (Figure 7).
Figure 7. TCA Precipitation for Albumin Depletion. Trichloroacetic acid (TCA) precipitation was used as a method to deplete the high abundance protein, albumin, in serum. A Coomassie Brilliant Blue stain was used on an SDS-PAGE gel to visualize the efficacy of the TCA method. 15 µg of protein was loaded on 4-20% SDS-PAGE gels. N/A = no protein precipitation. 1% = 1% TCA-IPA protein precipitation. 20% = 20% TCA-H₂O protein precipitation. 4% SDS (w/v): 100mM DTT, Tris-HCl pH 7.6; 8M urea: Tris-HCl pH 7.6. Albumin is shown at 75 kD. Both 4% SDS and 8M urea were tested as potential solvents.

Given our concerns about non-specific precipitation using the TCA method, we next used a commercially available albumin depletion kit from Pierce (P/N 85160), however the results of Coomassie Brilliant Blue staining on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels showed that there was still a large amount of albumin in the serum samples (Figure 8).

Next, we compared two commercially available depletion kits: the High Select HSA/Immunoglobulin Depletion Kit (Thermo Fisher Scientific, P/N A36365) and the High Select Top 14 Abundant Protein Depletion Kit (Thermo Fisher Scientific, P/N A36369). We compared the two kits again on an SD-PAGE gel using Coomassie Brilliant Blue staining (Figure 9). There was only a slight difference between the two kits on the gel, with the High Select HAS/Immunoglobulin Depletion Kit only ever-so slightly showing better depletion, so we opted
to use this kit in future experiments. To deplete samples, serum was pipetted over resin spin columns from the kit, washed three times, and combined. Protein assays were then carried out and samples were diluted 1:5-1:20 in H2O. For the assay, bovine serum albumin (BSA) was used as the standard and sample was loaded into the wells of a 96 well plate. Absorbance was read at 660 nm on a plate reader.

Serum protein was digested by filter-aided sample preparation (FASP). Samples were loaded onto FASP filters with 200µL of urea and centrifuged at 14,000 x g for 15 minutes and another 200µL of urea was added before samples were centrifuged again for 15 minutes. Proteins were incubated in the dark with 100 mM iodoacetamide (IAA) for 20 minutes and centrifuged once more for 15 minutes. Samples then received urea three times, followed by three additions of ammonium bicarbonate (ABC), and they were centrifuged again for 15 minutes. This step was repeated two more times before adding NaCl and centrifuging once more for 15 minutes. At the last step, 5 µL of formic acid was added.

Figure 8. Depletion of albumin in serum using a commercial kit. A commercial kit from Pierce (P/N 85160) was used for albumin depletion. The first lane is undepleted serum. The first band shows much less albumin as it was run through the depletion column. Columns 2-4 are the same sample being eluted again in order to see how many fractions were optimal. We retained fractions 1-4.
After digestion with trypsin, samples were desalted to remove any contaminants using C18 SPE columns that were placed under a vacuum manifold. One ml of acetonitrile (ACN) was pipetted over the columns and then 2mls of H₂O/0.1% formic acid (FA Sol) was added. The sample was loaded into the columns once they were equilibrated with ACN and FA Sol. Then the columns were washed and desalted with three additions of FA Sol. The sample peptides were collected in microcentrifuge tubes. Peptides then received two additions of 500 µL buffer (90:10 ACN:H₂O + 0.1% formic acid).

**Figure 9. Comparison of commercial HSA and Immunoglobulin versus Top 14 Abundant Protein depletion kits.** Two commercially available depletion kits: the High Select HSA/Immunoglobulin Depletion Kit (Thermo Fisher Scientific, P/N A36365) and the High Select Top 14 Abundant Protein Depletion Kit (Thermo Fisher Scientific, P/N A36369) were purchased and compared by Coomassie Blue Staining on SDS-PAGE gels. The HSA/Immunoglobulin kit showed slightly better depletion and was selected to be used for depletion in subsequent experiments. 5 µg of protein were loaded into each lane. SDS gel is a 4-20% gel (Bio-Rad).
LC-MS/MS

Peptides were separated using high performance liquid chromatography using a 50cm reversed-phase C18 UHPLC analytical column with an EASY-nLC1200 HPLC (Thermo Fisher Scientific). Peptides were subsequently analyzed on a hybrid-quadrupole-Orbitrap mass spectrometer (Q Exactive HF-X, Thermo Fisher Scientific) with a 120 minute gradient at the USF Morsani College of Medicine Proteomics Core Facility. Full MS survey scans were acquired at 60,000 resolution. Samples were analyzed in duplicate, the first time using the traditional DDA method, which selected the top 30 most abundant peptides (scan range 400-1600 m/z). The segmented DDA method selected the most abundant peptides across 3 ranges (375-600 m/z (top 20), 600-800 m/z (top 10), and 800-1200 m/z (top 10)). Raw data files were searched and combined.

Analysis

Raw data files were run through a search using MaxQuant software (Max Planck Institute of Biochemistry) against the Uniprot proteome database for the species Rattus norvegicus. Modifications included in parameters were cysteine by carbamidomethylation, variable modifications to methionine oxidation, and protein N-terminal acetylation. A 1% false discovery rate was used for protein and peptide identification. The LFQ feature was used for normalization.

Next, Perseus software (Max Planck Institute of Biochemistry) was used for filtering and imputation of data. LFQ intensities were input as expression values and then log-transformed. Data was filtered to only include proteins with LFQ intensities in at least 60% of samples within at least one treatment group. Any samples that showed a skewed, non-normal distribution following imputation were excluded from further analysis.

After Persues, data was exported to Microsoft Excel and ratios were generated between comparisons using the LFQ intensity values for each comparison. Additionally, Welch’s t-tests
were carried out in order to obtain p values for significance and z scores for directionality.

Proteins with a p < 0.05 were considered statistically significant.
Chapter 3 – Effects of Nutraceutical Intervention on Serum Proteins in Aged Rats

Abstract

Aging is associated with many pathophysiological changes that could lead to the onset of degenerative disease. Some of the physiological changes that occur with aging include increased inflammation and decreased stem cell proliferation, leading to decreased capacity for tissue regeneration and loss of function. In previous studies, we and others have found nutraceutical intervention to ameliorate some of the deleterious effects associated with aging. In particular, we have previously shown that NT-020, a supplement composed of a proprietary blend of blueberries, green tea, vitamin D3, and carnosine, is able to rescue age-related cognitive deficits, impaired neurogenesis, and inflammation in rats. We have also previously demonstrated that stem cells cultured with old serum showed decreased proliferation, however when stem cells were cultured in serum from old rats given a diet supplemented with NT-020, proliferation did not differ from that of cells cultured with serum from young rats. While it is clear that NT-020 is exerting a therapeutic, anti-aging effect, the mechanisms of action were yet to be fully elucidated.

To that end, in the present study, we conducted a bioinformatics experiment to examine the rat proteome of serum from young and old control rats, and young and old rats given a diet supplemented with NT-020.

Serum from old rats showed an increase in some inflammatory and pro-aging factors while serum from old rats given a diet supplemented with NT-020 showed an increase in some anti-aging factors, most notably proteins associated with the complement system and autophagy.
Introduction

As the population ages, there is increased susceptibility to the onset of disease and age-related degenerative conditions, making aging a central area of interest in medical sciences research (Wagster et al. 2012, Flowers et al. 2016). Aging is a complex and multifactorial physiological process associated with decreased stem cell proliferation in stem cell niches throughout the body, leading to reduced tissue rejuvenation and loss of organ function (Murshid, Eguchi and Calderwood 2013, Bickford et al. 2015). The decline in stem cell regeneration is global, occurring both in the periphery (Ning et al. 2019, Jung et al. 2019, Chambers et al. 2007) and in the central nervous system (CNS) (Villeda et al. 2011, Lazarov et al. 2010, Kozareva, Cryan and Nolan 2019, Ayaz et al. 2019, Kase et al. 2019, Stankiewicz et al. 2019, Bickford et al. 2015, Flowers et al. 2015, Acosta et al. 2010). While the exact biological mechanisms underlying dampened stem cell proliferation with age remain unclear, two areas of investigation are being explored: cell-autonomous changes and cell non-autonomous changes. Cell autonomous changes involve the senescence of genes such as p53, p16\textsuperscript{INK4A}, critical proteins involved in the Wnt/β-catenin pathways, and others (Sharpless and DePinho 2007, Bickford et al. 2015). Cell non-autonomous effects on stem cell niches refers to various factors, such as pro-inflammatory cytokines, chemokines, and other proteins that may influence the microenvironment (Bickford et al. 2015). Very salient early examples of circulating factors impacting stem cell proliferation come from heterochronic parabiosis studies. Heterochronic parabiosis entails the surgical union of the circulatory system of a young mouse with an old mouse. It was observed that exposure to blood from old mice had a deleterious impact on stem cell proliferation in young mice. Conversely, exposure to blood from young mice had a positive effect on stem cell proliferation in old mice, thus demonstrating how the systemic milieu can impact stem cell proliferation (Conboy et al. 2005, Villeda et al. 2011). Additionally, Villeda et al. (2011) injected plasma from either old or young mice into young mice four times over ten days.
and subjected the animals to contextual fear conditioning and the radial arm water maze. Young animals injected with plasma from old mice displayed significantly less freezing behavior in the contextual fear conditioning paradigm and made a greater number of errors in the radial arm water maze paradigm, suggesting impaired learning and memory following intravenous injection with old plasma. These studies further highlight how the aged systemic milieu can impact the CNS by exerting a dampening effect on hippocampal neurogenesis. In addition to these *in vivo* studies, the negative impact of the aged systemic milieu on stem cell proliferation has been reproduced *in vitro* with various types of stem cells derived from mice or rats being cultured with serum from old animals (Bickford et al. 2015, Villeda et al. 2014).

Many different factors from old blood have been identified as proteins of interest in terms of their potential to impact stem cell proliferation. In their initial study, Villeda et al. (2011) proposed that increased CCL11 (eotaxin) in old blood may be a key regulator impinging upon the cellular microenvironment. Another negative regulator that was proposed is beta2-microglobulin, a pro-aging factor (Smith et al. 2015). Additionally, at the same time, loss of protective factors has also been implicated in aging. Several studies have found that there is decreased expression of growth differentiation factor 11 (GDF-11) with age (Katsimpardi et al. 2014, Sinha, Patro and Patro 2019). Decreased expression of nuclear factor erythroid 2-related factor 2 (Nrf2) has also been observed with aging. Nrf2 is a transcription factor that regulates the expression of antioxidant proteins and, if it is downregulated with age, this is consistent with the known age-related increase in oxidative stress (Huang et al. 2019, Fulop et al. 2018, Flowers et al. 2016).

Polyphenols have been shown to rescue age-related dysfunctions within the CNS including chronic inflammation, impaired synaptic plasticity, and decreased neurogenesis (Ayaz et al. 2019, Flowers et al. 2015, Dias et al. 2012, Rahman, Biswas and Kirkham 2006, Vauzour 2012). Furthermore, polyphenolic compounds have also been shown to have beneficial effects
in mouse models of Alzheimer’s disease (AD) (Rezai-Zadeh et al. 2005, Rezai-Zadeh et al. 2009, Rezai-Zadeh et al. 2008, Singh et al. 2008). Our group has demonstrated that a proprietary blend of polyphenolic compounds including epigallocatechin gallate (EGCG) from green tea, blueberry extract, vitamin D3, and carnosine, called NT-020, attenuates inflammation, enhances hippocampal neurogenesis, and improves cognitive function in old rats (Flowers et al. 2015, Acosta et al. 2010). In a rat model of stroke, rats given an NT-020-supplemented diet showed increased neurogenesis compared to vehicle-treated rats (Yasuhara et al. 2008). In in vitro studies, NT-020 and the blue-green algae spirulina were shown to synergistically increase proliferation of human adult stem cells (Bachstetter et al. 2010, Shytle et al. 2010).

Furthermore, when rat mesenchymal stem cells (MSCs) or neural progenitor cells (NPCs) were cultured in serum derived from old rats, decreased proliferation was observed. However, if serum from old rats given a diet of chow supplemented with NT-020 was added to the culture, these effects were reversed (Bickford et al. 2015). Older adults given NT-020 supplementation in pill form in a double-blind placebo-controlled clinical trial demonstrated improved cognitive function on a battery of cognitive tests compared to participants in the placebo arm of the study, demonstrating the translational nature of nutraceutical research and effectiveness of NT-020 supplementation (Small et al. 2014). While some of the mechanisms of action for the therapeutic effects of NT-020 have been defined, such as the downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines, a complete profile of circulating factors has not yet been generated.

The goal of the current study was to generate a complete list of factors from the systemic milieu that could be altered as a consequence of aging and rescued by NT-020 supplementation. We used bottom-up, discovery-based mass spectrometry proteomics and a bioinformatics program called Ingenuity Pathway Analysis (IPA, Qiagen) to create a profile for
the entire proteome for serum derived from old rats given NT-020 or a normal diet and young rats given NT-020 or a normal diet. Our data suggest that there are age-related molecular changes that can be rescued by NT-020 supplementation.

Methods

Animals

To limit variability due to difference in sex that occur with age, male Fisher rats 3-6 months (young) or 20-22 months (old) of age were obtained from the National Institute on Aging (NIA) contract colonies and were housed at the University of South Florida (USF) AAALAC-accredited animal facility at the Morsani College of Medicine. All experimental procedures were approved by the institutional animal care and use committee (IACUC). The rats were fed either a standard NIH-31 chow or a NIH-31 chow supplemented with NT-020 (Natura Therapeutics, Inc.) ad libitum at 135 mg/kg/day for 28 days. Rats were divided into three experimental groups (n=10 per group): old rats given standard NIH-31 chow (old control), old rats given NT-020 supplementation (old diet), young rats given standard NIH-31 chow (young). Body weight and food consumption were monitored throughout the week and measured three times per week. No differences were observed in either body weight or food consumption for any of the groups. Rats were sacrificed by CO₂ euthanasia after 28 days. Blood was collected by cardiac puncture into serum collection tubes. Tubes were spun at 3000 rpm for 10 minutes and serum was then aliquoted and frozen at -80°C until use. Brains were also collected and stored in tubes at -80°C for use in a future study.

Sample Preparation

In order to prepare the serum samples for analysis on the mass spectrometer, the most abundant proteins had to be depleted from the samples. A volume of 10 µl of sample was added to each spin column containing resin filters from a High Select Top 14 Abundant Protein
Depletion Kit (Thermo Fisher Scientific, Inc., P/N A36369). Sample was pipetted over the resin spin columns from the kit, washed three times with wash buffer, and combined. Protein quantitation assays were carried out and samples were diluted 1:5-1:20 in H2O. Bovine serum albumin (BSA) was used as a standard and 10 µL of sample were added to each well of a 96 well plate. Pierce 660 Assay Reagent (150 µL) supplemented with ionic detergent compatibility reagent (IDCR) was added to the plate. Absorbance was read at 660 nm using a plate reader.

Serum protein samples were buffer exchanged and digested by filter aided sample preparation (FASP). Samples were loaded onto FASP filters with 200µl of urea buffer and centrifuged at 14,000 x g for 15 minutes. An additional 200µl of urea buffer was added to the sample reservoir and centrifuged again for 15 minutes. Proteins were then alkylated with 100 µL of 100 mM iodoacetamide (IAA) in the dark for 20 minutes, followed by centrifuging at 14,000 x g for 15 minutes. After alkylation, samples were buffer exchanged with three additions of urea, followed by three additions of ammonium bicarbonate (ABC), and then centrifuged at 14,000 x g for 15 minutes after each addition. Finally, proteins were digested by adding Trypsin/Lys-C in a 1:50 ratio of trypsin:protein (w:w) and incubated overnight at 37°C. After incubation, peptides were collected by adding 40 µL of ABC buffer and centrifuging at 14,000 x g for 10 minutes. This was repeated for a total of two additions of ABC, before adding 50 µL of NaCl and again centrifuging for 15 minutes. Lastly, 5 µL of formic acid was added to acidify the samples before desalting.

Following trypsin digest, peptide samples were desalted using C18 SPE columns placed on a vacuum manifold. The C18 SPE columns were activated with one ml of acetonitrile (ACN), and then equilibrated with two volumes of H2O/0.1% formic acid (FA Sol). Once equilibrated, sample was loaded onto the C18 SPE columns, and then washed/desalted with three volumes of FA Sol. The flow-through collection tubes were then replaced with microcentrifuge tubes for
peptide collection. Peptides were eluted with two additions of 500 µL of elution buffer (90:10 ACN:H₂O + 0.1% formic acid). Samples were dried completely in a vacuum concentrator and then resuspended in 1%ACN/99%H₂O/0.1% formic acid.

LC-MS/MS

Peptides were separated on a 50cm reversed-phase C18 UHPLC analytical column using an EASY-nLC 1200 HPLC (Thermo Fisher Scientific) and analyzed on a hybrid-quadrupole-Orbitrap mass spectrometer (Q Exactive HF-X, Thermo Fisher Scientific) with a 120 minute gradient. Full MS survey scans were acquired at 60,000 resolution. Each sample was analyzed twice, once using a ‘traditional data-dependent acquisition (DDA)’ method, and once using a ‘segmented DDA’ method. The ‘traditional DDA’ method selected the top 30 most abundant peptides across the full mass scan range of 400-1600 m/z. The ‘segmented DDA’ method selected the most abundant peptides across 3 different mass scan ranges: 375-600 m/z (top 20), 600-800 m/z (top 10), and 800-1200 m/z (top 10). Raw data files for each method were searched as technical replicates and combined.

Analysis

Raw data files were searched using MaxQuant software (Max Planck Institute of Biochemistry) against the current *Rattus norvegicus* proteome database from Uniprot. Parameters included the fixed modification of cysteine by carbamidomethylation, as well as the variable modifications methionine oxidation and protein N-terminal acetylation. A 1% false discovery rate was used for both peptide and protein identification. The MaxQaunt LFQ feature was used for label free normalization.

Perseus software (Max Planck Institute of Biochemistry) was used for the filtering and imputation of proteomics data as described previously (Tyanova et al. 2016). Briefly, label free quantification intensities (LFQ) were used as expression values and log transformed. The data
was filtered to include proteins that were identified with LFQ intensities (valid values) in at least 60% of samples within at least one treatment group, and missing values were then imputed using the default settings. Samples that showed a skewed, non-normal distribution following imputation were excluded from further analysis. The following number of samples remained per treatment group: young control (n=6), old control (n=7), and old diet (n=5).

Following imputation in Perseus, the data was exported and Excel was used to generate ratios between comparisons, as well as calculate Welch’s t-test p-values, and z-scores, as previously described (Flowers et al., 2017). Briefly, ratios were generated using the LFQ intensity values for each comparisons of interest. Proteins with a Welch’s t-test $p < 0.05$, determined using log2-transformed LFQ intensities, were considered statistically significant.

**Results**

We detected a total of 210 proteins in all samples. There were 40 “unmapped” proteins that were not identified by Persius. A full list of identified proteins is included (Table 3). We then examined the proteins of interest that were found to be significantly different in the pairwise comparisons of the groups.

**Change of Protein Expression as a Function of Age**

Complement C1r was significantly decreased in serum from old rats compared to young ($p < 0.05$, Table 1). In addition, complement proteins complement 5 (C5), complement 6 (C6), and complement 8 gamma chain (C8) decreased with age ($p < 0.05$, Table 1). The protein ceruloplasmin, which is a copper transporter and antioxidant protein, was also found to be significantly decreased ($p < 0.05$, Table 1), which is consistent with the literature showing an age-dependent decrease in this protein and, interestingly, a decrease in this enzyme is associated with neurodegeneration (Musci et al. 1993, Connor et al. 1993, Jeong and David 2006). There was also a significant decrease in expression of insulin like growth factor binding
protein acid labile subunit (IGFALS) (p < 0.05, Table 1). It is interesting to note that with osteoporosis, there is a decrease in IGF-1 and its binding proteins in the serum (Fritton et al. 2010). Additionally, there was significantly decreased expression of maltase-glucoamylase (Mgam), which is consistent with age-dependent impaired digestion of carbohydrates (Fernandez-Alarcon et al. 2017).

In order to query how these changes affect functional pathways, the data were uploaded into Ingenuity Pathway Analysis (IPA, Qiagen) and the p value cutoff extended to P<0.1 in order to increase the number of proteins for functional associations. The changes can be viewed graphically in Figure 10A. This figure illustrates the changes with the highest Z score all of which were related to immune function. Five cellular functions were top hits and they are shown at the center of the figure. The proteins driving these findings are illustrated on the outside of the circle. The fold change is listed below each protein, green indicates a decrease and red an increase. As illustrated all of the functional predictions are for an upregulation of immune responses including activation of macrophages and phagocytes.

**NT-020 Counteracts Upregulated Immune Response in Aged Rat Serum**

We next compared changes in the old animals following treatment with NT-020. All of the proteins that were entered into IPA for Fig 10A were also uploaded for the ratios in the old diet treated rat serum versus old control diet, to explore how diet altered the expression ratio. Only 7 of the 31 proteins that were significantly changed with age, were significant different with diet treatment at p<0.05 and these are discussed below. For comparison with the change with age in immune function pathways we illustrate the same function pathways in Figure 10B with the same set of proteins used to generate Figure 10A. As is observed in Fig. 10B, all of the immune functional pathways that were upregulated with age are down regulated in the aged rats on the NT-020 diet. For example, cystatin 3 (CST3) changed from 3-fold increase with age to a -1.6 decrease with diet treatment. CD5L was increased 2.2-fold with age and decreased
1.9-fold with NT-020 diet treatment. Overall this led to a predicted functional down regulation of the immune response with NT-020 diet in the old rats.

**Autophagy Pathway Protein Cathepsin A is Upregulated with NT-020**

The autophagical protein cathepsin A (CTSA) was shown to be significantly increased in serum from old rats given a diet supplemented with NT-020 (p<0.05, Table 2). Autophagy is a turnover process for protein aggregates and damaged organelles. This process is considered protective and anti-aging, and inhibition or loss of autophagy occurs with age. Compromised autophagy has also been linked to neurodegenerative diseases and metabolic defects that are associated with aging (Martinez-Lopez, Athonvarangkul and Singh 2015, Boland et al. 2018). Autophagy is driven by a number of different proteins, among which are cathepsins. CTSA is a lysosomal protein that specifically triggers chaperone-mediated autophagy (CMA) through its serine carboxypeptidase activity, which triggers the degradation of lysome-associated protein type 2a (Cuervo et al. 2003).

**Macrophage Stimulating 1 Decreases with NT-020**

Macrophage stimulating 1/macrophage stimulating protein/hepatocyte growth factor-like protein (MST1/MSP/HGFL) expression was found to be significantly decreased with NT-020 treatment in old rats compared to old control (p<0.05, Table 2). MST1 is a protein in serum that is predominantly secreted by liver hepatocytes. Some studies have pointed to MSP as a negative regulator of inflammation and a key protein in maintaining metabolic homeostatis (Li et al. 2015). However, there is evidence that MST1/MSP activation increases pro-inflammatory cytokine production in the liver and lungs (Li et al. 2016, Wang et al. 2015). In the CNS, it has been found that there are receptors for MSP on microglia (Ron) and that MSP activation results in increased mRNA expression of pro-inflammatory cytokines (Suzuki et al. 2008).

**Complement System Proteins were Altered by NT-020**
The complement system is part of the innate immune response and is considered protective for the retina with aging (Mukai et al. 2018). However, it has also been proposed that complement C1q promotes aging through interaction with the canonical Wnt signaling pathway (Naito et al. 2012). Additionally, early components of the complement system have been shown to increase with age in a mouse model of AD (Reichwald et al. 2009). There are many proteins involved in the complement system, but two were found to be altered by NT-020: serpin G1, which increased with NT-020 treatment in old rats, and complement factor H (CFH), which decreased with NT-020 treatment in old rats (p < 0.05, Table 2). Both of these changes are consistent with an improved complement function. Serpin G1, also called C1 inhibitor or C1 esterase inhibitor, is largely considered to be a protective factor and its inhibition has been associated with impaired blood-brain barrier (BBB) integrity and neuroinflammation in the brain (Farfara et al. 2019). Furthermore, deficient C1 inhibitor expression is linked to hereditary angioedema (Mete Gokmen et al. 2019), further suggesting a protective function.

Complement factor H (CFH) is an important regulator of the alternative pathway of the complement system. Interestingly, increased plasma CFH has been found to be correlated with geriatric depression (Shin et al. 2019). Additionally, genetic variants of CFH are strongly associated with age-related macular degeneration (AMD) and CFH has been shown to inhibit the anti-inflammatory activity of CD47 (Calippe et al. 2017). It has also been found that increased concentrations of complement 3 and CFH in the cerebrospinal fluid (CSF) of Parkinson’s disease, Alzheimer’s disease, and multiple-system atrophy patients correlated with disease severity (Wang et al. 2011).

Discussion

Aging involves multiple complex physiological changes that lead to increased inflammation, loss of homeostasis and resilience, and impaired regenerative capacity. The role of inflammation in aging has been widely researched and the term “inflammaging” has been
coined to summarize how the inflammatory milieu leads to degeneration (Minciullo et al. 2016). While there is an increase in pro-inflammatory and other pro-aging factors and a decrease in anti-inflammatory factors with age, a complete profile of the aging milieu has yet to be identified, although parabiosis studies have contributed significantly to this effort (Villeda et al. 2011, Villeda et al. 2014, Smith et al. 2015). The current study sought to identify factors that were significantly changed with age and could be ameliorated by treatment with the polyphenol-rich dietary supplement NT-020, as NT-020 has been demonstrated to have beneficial effects in vitro and in vivo (Yasuhara et al. 2008, Shytle et al. 2010, Bachstetter et al. 2010, Kaneko et al. 2012, Small et al. 2014, Bickford et al. 2015, Flowers et al. 2016).

Eleven proteins were found to change significantly when comparing old control rats to young controls. Of these, ceruloplasmin, insulin like growth factor binding protein acid labile subunit (IGFALS), and maltase-glucoamylase (Mgam) were the most noteworthy. The protein ceruloplasmin, which is an enzyme that functions as a copper transporter and antioxidant protein, decreased significantly with age, which would be expected and, interestingly, a decrease in this enzyme is associated with neurodegenerative diseases (Musci et al. 1993, Connor et al. 1993, Jeong and David 2006). There was also a significant decrease in expression of IGFALS and decreased expression of IGF and its binding proteins in serum has been linked with increased adipogenic potential of mesenchymal stem cells over osteogenic potential in osteoporosis (Fritton et al. 2010). Additionally, there was a significant decrease in expression of Mgam, which is consistent with age-dependent impaired digestion of carbohydrates and susceptibility to type 2 diabetes (Fernandez-Alarcon et al. 2017, Ren et al. 2011). Collectively, these data suggest that the significantly changed proteins were impacted by age as expected. However, complement proteins were observed to decrease with age, whereas in the literature they have been shown to increase with age (Gaya da Costa et al. 2018). While the reason for this disparity is unclear, the previously discussed proteins changed as expected.
with age. Further study is needed to determine whether this trend was global or tissue-specific, and a future study is ongoing, as later discussed.

Interestingly, when the data were entered into IPA and the p value cutoff extended to p<0.1 in order to increase the number of proteins identified and query functional pathways, the results showed that the proteins identified drove upregulation of immune responses such as activation of macrophages and phagocytes with age and that all of these functional pathways became downregulated in the old NT-020 diet group. These data suggest that NT-020 was able to dampen the immune response in old rats.

Autophagy is the pathway by which protein aggregates and damaged organelles are degraded. With aging, there is decreased autophagy, leading to increased aggregation of proteins, which results in a toxic microenvironment and cellular dysfunction. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Altogether, autophagy is critical for protein and cellular homeostasis. The exact mechanisms underlying decreased autophagy with aging are unclear, however it has been noted that autophagy is decreased in aging stem cell niches in particular, contributing to decreased regenerative capacity (Revuelta and Matheu 2017). There are many proteins that are involved in the autophagy pathway and among them are cathepsins. In the current study, cathepsin A (CTSA), which triggers CMA (Cuervo et al. 2003), was found to be increased with NT-020 treatment in old rats compared to age-matched controls.

Macrophage stimulating 1/macrophage stimulating protein/hepatocyte growth factor-like protein (MST1/MSP/HGFL) is a serum protein that is primarily secreted by hepatocytes in the liver. This protein has been previously speculated to have protective effects and to be a necessary regulator of metabolic activity (Li et al. 2015). However, recent studies have shown that MST1/MSP/HGFL increases pro-inflammatory cytokine production in the liver and lungs (Li et al. 2016, Wang et al. 2015). Non-alcoholic steatohepatitis (NASH) is a liver disease
characterized by inflammation and accumulation of lipids in the liver. NASH model mice treated with MSP were found to have significantly higher levels of tumor necrosis factor-α (TNF-α), chemokine (C-C motif) ligand 2 (Ccl2), intracellular adhesion molecule 1 (Icam1), interleukin 1β (IL-1β), interferon gamma (IFNγ), B cell lymphoma 2 (Bcl2), macrophage markers F4/80, and cluster of differentiation 68 (CD68) (Li et al. 2016). Wang et al. exposed rats to combustion smoke in order to create a model of smoke-induced airway inflammation and found that MST1/MSP/HGFL and its receptor, RON, stimulated production of pro-inflammatory cytokines TNF-α, IL-8, IL-1β, and IL-10 (Wang et al. 2015). In the current study, MST1/MSP/HGFL decreased when old rats were given a diet supplemented with NT-020 compared to age-matched controls. Given the role of this protein in inflammation, this finding shows a mechanism by which NT-020 may resolve aspects of age-related inflammation.

The complement system constitutes part of the innate immune response. Some studies have shown that the complement system is protective against age-related retinal degeneration (Mukai et al. 2018). However, another study has shown that complement protein C1q promotes aging through activation of the canonical Wnt pathway (Naito et al. 2012). Furthermore, Reichwald et al. showed that expression of early complement proteins increased in an age-dependent manner and may contribute to AD pathogenesis in amyloid precursor protein-overexpressing mice (Reichwald et al. 2009). Together these findings suggest that proteins from the complement system should be analyzed individually as to whether or not they are contributing to the aging phenotype. In the current study, we found changes in the complement proteins serpin G1/C1 inhibitor/C1 esterase inhibitor and CFH. Specifically, we observed a significant increase in the protective protein serpin G1 in old rats treated with NT-020. Knockdown of circulating plasma C1 inhibitor in the brain has led to decreased BBB-mediated extravasation and infiltration of plasma proteins and immune cells, activation of glial cells, impaired cognition, and depressive behavior in mice (Farfara et al. 2019). Interestingly, we also
observed a significant decrease in CFH in serum from old rats given a diet supplemented with NT-020 compared to old rats given a standard diet. CFH is critical for activation of the alternate complement system. In a recent study, increased levels of CFH in plasma was positively correlated with depression in geriatric patients (Shin et al. 2019). Genetic variants of the CFH gene, which encodes the CFH protein, are associated with age-related macular degeneration (AMD). Calippe et al. also demonstrated that one AMD-associated CFH variant was able to suppress the anti-inflammatory activity of CD47 (Calippe et al. 2017). Another study examined cerebrospinal fluid (CSF) samples taken from patients with Parkinson’s disease, Alzheimer’s disease, and multiple-system atrophy and found that complement 3 (C3) and CFH were both increased, though to varying ratios depending upon the disease. Interestingly, both C3 and CFH concentrations correlated with disease severity in AD (Wang et al. 2011).

The results of the current study suggest that NT-020 is targeting multiple mechanisms associated with aging including autophagy and the complement system. However, the study was not without limitations. In spite of using a commercial kit to deplete the highly abundant albumin in our serum samples as well as the other most abundant proteins in serum, our detection of a low number of proteins suggests that albumin and other high abundance proteins remained, preventing detection of other proteins in the samples. One reason for this could be that the depletion kit used was optimized for human tissue and not rat, however there are no commercially available depletion kits with specificity for rat protein. Prior to opting to use this kit, we attempted to use an acid precipitation method, but found it to be non-specific, leaving the possibility that we could be losing proteins we didn’t wish to deplete from the samples as well. Thus, we opted to use the human-optimized kit from Thermo Fisher Scientific, Inc.

It is additionally worth noting that the proteins with a significant fold change that were detected in the old rats were not the same proteins found to have a significant fold change in the old rats given a diet supplemented with NT-020. Therefore, there was no detection of differential
expression of individual proteins between these two groups. However, there were common pathways between them, most notably the complement system.

In addition, upon collection of serum, a protease inhibitor was not added prior to storing the samples at -80°C before use. This could also have led to protein degradation prior to analysis. Protease inhibitor was added after samples were thawed and processed for mass spectrometry, however this step may have been undertaken later than would have been optimal.

In spite of some of the methodological challenges involved with working with serum, these findings contribute to the existing body of knowledge on how nutraceuticals impact the aging milieu and exert therapeutic effects. In particular, we have found that NT-020 reverses the negative effects of age on stem cell proliferation, enhances neurogenesis, and leads to improved performance on cognitive behavioral paradigms (Acosta et al. 2010, Bachstetter et al. 2010, Bickford et al. 2015, Flowers et al. 2015, Flowers et al. 2016, Kaneko et al. 2012, Shytle et al. 2010, Small et al. 2014, Yasuhara et al. 2008). These data provide insight into the mechanisms of action underpinning NT-020-mediated therapeutic effects. Additionally, future studies are needed to compare the proteome from serum to that of CNS tissue or cells in order to determine whether circulating factors in the periphery have an impact on the brain. A study is currently in progress to compare the results of the serum analysis to microglia from the same animals.
### Table 1. Expression of proteins in old rat serum compared to young

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<td>Complement C5</td>
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<td>C8G</td>
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<td>Ceruloplasmin</td>
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<td>Carboxypeptidase N subunit 2</td>
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### Table 2. Expression of proteins in old NT-020 serum versus old control

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### Table 3. Complete list of proteins

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<th>Welch’s t-test Old Diet/Old Control</th>
<th>Z-score O-D vs O-C</th>
<th>Ratio Old Ctl/Young Ctl</th>
<th>Welch’s t-test Old Ctl/Young Ctl</th>
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Table 3 continued

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Figure 10. Predicted Activation of Cellular Immune Functions with Age. As can be seen in this graphic representation of the predicted activation (orange) or inhibition (blue) of cellular immune functions, clustered in the middle of the graphic. In panel A there is predicted upregulation of phagocytes, neutrophil adhesion, macrophages, binding of phagocytes and immune response that is driven by the
proteins detected in the old rat serum compared to young rat serum as shown in panel A. The proteins driving these predicted changes in cellular immune function are shown surrounding the functions. The fold change for protein expression is written below each protein along with the -Log p value. As shown in the insert to the right, red indicates increased expression and green indicates decreased expression and the intensity of the color reflects the degree of expression. For example, A2M is upregulated 1.75-fold in the aged rat serum and CST3 is upregulated 3.11-fold. In panel B the same proteins used in panel A were entered into IPA for the old vs old NT-020 treated comparison. As can be seen the changes in protein expression following treatment with NT-020 now show a predicted inhibition of these same pathways in the old treatment group. For example, A2M is not -1.24 and CST3 is -1.62-fold. This shows an overall reduction in immune function predicted by the pattern of protein expression detected in the aged rat serum in the NT-020 treated animals. Abbreviations: apolipoprotein E (APOE), complement component 3 (C3), SERPINF2, SERPING1, complement factor H (CFH), cystatin 3 (CST3), SERPINA1, alpha-2-macroglobulin (A2M), CD5 molecule like (CD5L), angiotensin (AGT), serum amyloid P component (APCS).
Chapter 4 – Conclusion and Future Directions

Conclusion

Overview

Aging is the main risk factor for cancer, cardiovascular disease, and neurodegenerative diseases (Niccoli and Partridge 2012). As the life expectancy of the population continues to increase, the need to address diseases that impact health span and quality of life for the elderly continues to increase (Crimmins 2015, Centers for Disease Control and Prevention 2019). With aging there is a progressive decline in organ function and homeostasis throughout all biological systems in the body, leading to decreased cell proliferation and regeneration (Wang, Karpac and Jasper 2014, Oh, Lee and Wagers 2014). The age-related decrease in renewal ability of stem cells and the overall decline in function of stem cell niches, in particular, underlies the reduced capacity for rejuvenation and repair (Ahmed et al. 2017).

While the exact mechanisms underlying aging are largely unknown, recent investigation has focused on cell autonomous changes and cell non-autonomous changes. Cell autonomous changes include senescence of genes and loss of function of critical proteins. Cell non-autonomous changes include effects on stem cell niches such as oxidative stress, pro-inflammatory cytokines, chemokines, and other signaling and environmental factors that can impinge on and influence the microenvironment (Bickford et al. 2015, Cho et al. 2013, Demaria et al. 2015). A lot of evidence for the cell non-autonomous effects on the aging microenvironment have been provided by parabiosis studies. In these studies, heterochronic pairs are created by surgical union of the circulatory system of a young animal with an old animal (Villeda et al. 2011, Villeda et al. 2014).
Previous studies have shown that old heterochronic rats have increased neurogenesis in the subgranular zone of the dentate gyrus in the hippocampus compared to age-matched isochronic (control) animals not exposed to young blood. Additionally, heterochronic young rats have decreased neurogenesis in the hippocampus compared to age-matched isochronic animals not exposed to old blood. Additionally, young mice injected with plasma from old mice made a greater number of errors in the radial arm water maze (RAWM) paradigm and displayed significantly less freezing behavior on the contextual fear conditioning paradigm than age-matched controls, indicating decreased cognitive ability in young mice injected with old plasma (Villeda et al. 2011, Villeda et al. 2014, Smith et al. 2015). In addition, graft transplantation studies have shown that age can have a negative effect on graft survival and this can be ameliorated by dietary supplementation (Willis et al. 2008, Willis et al. 2010). Together, these data indicate that there are negative factors in old blood and positive factors in young blood. This suggests that what may be driving aging is the loss of protective, rejuvenating factors from the microenvironment and the emergence of deleterious, pro-aging factors.

**Aging and NT-020**

Given the evidence of decreased stem cell proliferation with aging and the emergence of non-autonomous influences on the microenvironment, such as oxidative stress, it would seem that an approach for quelling the negative effects of aging would be a method for increasing endogenous stem cell proliferation. Previous studies by our group have shown that the nutraceutical compound NT-020, a proprietary blend of blueberry, green tea, carnosine, and vitamin D3, has increased proliferation of CD34+ cells in culture (Bickford et al. 2006). Additionally, rats that were prophylactically treated with NT-020 by oral gavage prior to MCAo stroke surgery had improved outcomes after stroke compared to vehicle-treated control stroke rats (Yasuhara et al. 2008). In aged rats, oral gavage with NT-020 also resulted in increased neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and
in the subventricular zone (SVZ) compared to age-matched vehicle-treated controls. Old NT-020-treated rats also made fewer errors on the Morris Water Maze paradigm than age-matched controls, showing evidence of improved cognitive performance (Acosta et al. 2010).

Furthermore, when NT-020 was used in a double-blind placebo-controlled clinical trial, older adults using the NT-020 supplement demonstrated improvement on two measures of processing speed in cognitive testing and it was shown that NT-020 was safe and well-tolerated, supporting NT-020 as a promising treatment for future clinical studies (Small et al. 2014). Additional pre-clinical work using NT-020 has shown that NT-020 has recapitulated Wnt signaling, which decreases with age, in old animals. Old rats maintained on a diet supplemented with NT-020 additionally had significantly lower concentrations of pro-inflammatory cytokines than old rats maintained on standard NIH-31 chow (Flowers et al. 2015, Flowers et al. 2016). Additionally, it has been demonstrated in vitro that neural progenitor cells (NPCs) and mesenchymal stem cells (MSCs) treated with serum from old rats had significantly decreased proliferation compared to cells treated with serum from young rats. However, compared cells treated with serum from old control rats, there was significantly greater proliferation when cells were treated with serum from rats maintained on a diet supplemented with NT-020 (Bickford et al. 2015).

Serum Data

The previous data from studies with NT-020 demonstrate a positive effect on neurogenesis and cognition, however the precise mechanisms underlying the observed benefits were not entirely clear. We therefore designed a bottom-up, discovery-based, mass spectrometry-based bioinformatics study to profile the serum proteome of aged (20-22 months of age) and young (3-6 months of age) rats maintained on a standard NIH-31 chow or an NIH-31 chow supplemented with NT-020. Rats were maintained on their respective diet for 28 days
prior to sacrifice and blood collection. Serum was isolated from blood and used for mass spectrometry.

Serum was depleted with a commercial depletion kit to remove high abundance proteins prior to further processing and analysis. As previously mentioned, the kit used to deplete albumin and IgG was optimized for human, as are all commercially available depletion kits. The lack of specificity for the species for which it was being used could have resulted in incomplete depletion of high abundance protein from samples, which could have impacted the MS results.

We detected a total of 210 proteins in all samples, and there were 40 “unmapped” proteins that were not identified by Perseus software. Of the proteins detected, there were 11 found to be significantly different between the old control rats and the young control rats and there were 7 found to be significantly different between the old diet versus the old control group. However, we did not find that there was differential expression of any of the same proteins between any of the treatment groups. A full list of the proteins identified is available in Table 3.

Of the proteins found to be significantly different in the pairwise comparison of the old control versus the young control, we noted changes in complement proteins complement C1R, complement 5 (C5), complement 6 (C6), and complement 8 gamma chain (C8), all of which decreased with age ($p < 0.05$, Table 1). The copper transporter, ceruloplasmin, also significantly decreased with age ($p < 0.05$, Table 1), which in particular is consistent with findings showing age-related decreases in expression levels of this protein (Musci et al. 1993, Connor et al. 1993). There were also significant decreases in insulin-like growth factor binding protein acid labile subunit (IGFALS) and maltase-glucoamylase (Mgam), which are both associated with age-dependent decline (Fritton et al. 2010, Fernandez-Alarcon et al. 2017).

As mentioned, there were only 7 proteins found to be significantly different in the pairwise comparison between the old diet versus the old control groups. Of these proteins, the most biologically relevant in terms of the anti-aging effect of NT-020 were the auotophageal
protein cathepsin A (CTSA), macrophage stimulating 1 (MST1 also referred to in the literature as macrophage stimulating protein (MSP) and hepatocyte growth factor-like protein (HGFL)), and the complement proteins serpin G1 and complement factor H (CFH).

**Autophagy and Aging**

Autophagy is an important degradation process for the clearance of proteins from the cytoplasm. There are different subtypes of autophagy, including macroautophagy, microautophagy, and chaperone-mediated autophagy, but all of these processes involve the degradation of proteins in the lysosome. Most of the literature focused on aging has centered on macroautophagy in which the cell sequesters a part of its cytoplasm by forming an autophagosome, which merges with the lysosome and degrades. Autophagy is considered a maintenance and self-protective process and inhibition of autophagy is associated with aging. There are age-related neurodegenerative diseases associated with the aggregation of proteins, such as α-synuclein in Parkson’s disease (PD), tau in Alzheimer’s disease (AD), and mutant huntingtin protein in Huntington’s disease. One of the functions of autophagy is to prevent these aggregates from forming. Autophagy also removes dysfuctioning mitochondria to prevent any pro-apoptotic signaling or oxidative stress. In these ways, autophagy functions to maintain homeostasis (Rubinsztein, Marino and Kroemer 2011). Autophagy has cell non-autonomous anti-aging effects including moderating the inflammatory response in several ways. Autophagy is required for the clearance of dying cells, thus reducing inflammation by removing debris. Autophagy also maintains intracellular levels of ATP, which produce lysophosphatidylcholine and phosphatidylserine which further facilitate clearance of dying cells, avoiding a secondary immune response.

As previously mentioned, autophagy can clear dysfuctioning mitochondria, thus preventing the production of ROS (Rubinsztein et al. 2011). Autophagy is driven by a number of different proteins, including cathepsins. CTSA is a
lysosomal protein integral to the autophagelal pathway in that it triggers chaperone-mediated autophagy (CMA) through its serine carboxypeptidase activity. CMA is a process by which damaged and malfunctioning intracellular proteins are degraded in lysosomes. Reduced CMA is also associated with neurodegenerative diseases, including PD and AD (Cuervo and Wong 2014, Cuervo et al. 2003).

**Macrophage Stimulating 1 and Complement Proteins**

Macrophage stimulating 1 (MST1) was also found to be significantly decreased in old animals maintained on an NT-020 diet compared to age-matched controls. MST1 is a serum protein that is predominantly expressed in hepatocytes and functions to upregulate inflammation by stimulating the release of pro-inflammatory cytokines (Wang et al. 2015, Li et al. 2016, Suzuki et al. 2008). As previously mentioned, inflammation is central to hypotheses of aging so this finding was of particular interest.

The complement system is part of the innate immune response. It has been suggested that the complement system is protective for the retina with aging (Mukai et al. 2018), however C1q may promote aging though its interaction with the canonical Wnt signaling pathway (Naito et al. 2012) and early components of the complement system have been associated with the pathogenesis of AD (Reichwald et al. 2009). The complement proteins found to change with NT-020 diet in old rats compared to age-matched controls were serpin G1, which increased with NT-020, and complement factor H (CFH), which decreased with NT-020. The inhibition of serpin G1 has been linked to loss of blood-brain barrier integrity and neuroinflammation (Farfara et al. 2019). CFH is a regulator of the alternative pathway for the complement system and its upregulation has been associated with geriatric depression, increased inflammatory activity, and age-related macular degeneration (Shin et al. 2019, Calippe et al. 2017). Furthermore, increased concentrations of CFH have been found in the cerebrospinal fluid of PD, AD, and multiple-system atrophy patients (Wang et al. 2011). There is conflicting information on how the
complement system behaves (whether its activity increases or decreases) with age and whether the complement system is protective with aging or whether it contributes to age-related decline and disease. It may be more useful to focus on the individual proteins than the system as a whole, as the evidence obtained in the current study is what would be expected from the literature on these two proteins.

**Apolipoproteins**

In addition to the proteins discussed, apolipoprotein B (apoB), apolipoprotein C3 (apoC3), apolipoprotein C4 (apoC4), and apolipoprotein N (apoN) also significantly increased in serum from old rats maintained on an NT-020-supplemented diet compared to age-matched controls. Age-related increases in apoB in serum from senescent mice have been observed (Hiuchi et al. 1992). Furthermore, age-related increases in apoB are associated with risk for developing cardiovascular disease (Sniderman et al. 2016). The role of apoC3 in aging is not well defined. There is some evidence of its decreased function negatively impacting mobilization of lipids (Araki, Okazaki and Goto 2004). There is also evidence from a study with centenarians and their offspring, that polymorphisms of the APOC3 gene confer a favorable lipoprotein profile, promote a healthy cardiovascular system, and promote longevity (Atzmon et al. 2006). The roles of apoC4 and apoN in aging and disease are not documented.

The data from individual proteins suggest an overall positive effect of NT-020 in aged rats, and the data in the current study support the anti-aging effects of NT-020. With aging there is an overall shift in cellular and molecular signaling towards inflammation and senescence and away from processes that promote cellular homeostasis and regeneration (Hartl 2016). The current study supports that a nutraceutical could correct this course and shift the microenvironment back towards a more homeostatic state by promoting processes such as autophagy, anti-inflammation, and antioxidant activity to combat oxidative stress. Furthermore, as suggested by the data with NT-020, many nutraceuticals are considered to possess multiple
health benefits, thus making it possible they can target myriad processes related to aging (Das et al. 2012).

With the growing age of the population increasing healthcare expenditure, nutraceuticals continue to be an area of interest for their ability to not only confer benefits for chronic conditions, but also to be used prophylactically in order to prevent the onset of chronic disease states (Nasri et al. 2014). Use of a nutraceutical in combination with exercise has also been shown to prevent neurodegeneration (Malaguti et al. 2019). Informing the public of lifestyle choices as a way of promoting healthspan is a valuable way to perhaps empower people to make healthy choices that can enhance their quality of life as they enter late adulthood.

**Future Directions**

**Study Limitations**

As previously mentioned, the data from individual proteins, though not differentially expressed in the old control versus young control compared to the old diet versus old control groups, is promising in that it shows a trend towards anti-inflammation, pro-autophagy, and a general anti-aging effect with NT-020 treatment in old animals, which is consistent with the existing body of data on NT-020. However, the overall lack of significant results obtained makes it difficult to draw any major conclusions without follow-up studies.

There are some potential methodological reasons for the small number of significant proteins in the dataset. As mentioned earlier in the overview subsection, the commercially available depletion kit from Thermo Fisher Scientific was optimized for human serum, as are all other commercially available depletion kits. This makes working with serum from other species for mass spectrometric analysis particularly challenging. Incomplete or insufficient depletion of high abundance proteins in a sample can interfere significantly with detection of lower abundance proteins (Han et al. 2008).
Additionally, there are challenges already “baked in” to mass spectrometry itself. MS analysis inherently preferentially samples peptides at high abundance over those of low abundance. According to the Broad Institute, LFQ inextricably related to abundance and results can vary depending upon instrument settings and the number of peptides in a given protein. LFQ is considered reliable only for highly abundant proteins and the results can produce a lot of missing data and variability, particularly for lower abundance proteins (Carr). The challenges presented by lack of species specificity in the depletion kit as well as MS being inherently better for analytes with high abundance peptides make it highly likely that lower abundance peptides in the serum samples were present, but went undetected due to preferential sampling of the high abundance peptides.

There were also some technical issues present when isolating the serum from the blood by centrifugation. Some serum samples had a slight pink hue, indicating hemolysis of red blood cells in the serum. Additionally, protease inhibitor was not added before freezing and storing the serum samples. Protease inhibitor was added at a later stage, during processing for MS, but the failure to add it before freezing and storing may have resulted in some protein degradation.

Given the promising results of what proteins did show significance in the results, future studies should be undertaken to overcome some of these methodological issues.

Future Studies

NT-020 was shown to be safe and well-tolerated in older adults in a double-blind, placebo-controlled clinical trial (Small et al. 2014). One of the methodological limitations of the current study was that serum depletion kits, which are necessary for MS studies with serum, are all optimized for human serum and there are none available for murine studies. As NT-020 has been shown to be safe and produced promising results, it may be beneficial to design another clinical trial and, this time, retain some of the blood collected to isolate serum for MS.
As one of the limitations discussed in the clinical study by Small et al (2014), was a lack of robust results on cognitive tests because all older adult participants were typically functioning and not impaired, it would be prudent to include a group of participants with clinical age-related cognitive deficits, such as mild cognitive impairment (MCI).

Future studies should further explore the roles of autophageal proteins, complement proteins, and other mediators of inflammation on aging and ascertain whether a polyphenol-rich supplement exerts an overall anti-aging effect. Discovery-based studies should query the entire human serum proteome for a full list of proteins that change with age and proteins that change with diet. Overall the current study is suggestive of an effect on proteins in the autophagy pathway and complement proteins in particular, indicating further potential anti-aging effects of NT-020. This study provides further support to the existing body of literature indicating that lifestyle, namely diet and exercise, contributes to healthy aging through dampening of inflammatory pathways as well as augmentation of anti-aging pathways. In particular, it has been observed that high antioxidant diets combined with physical activity stimulate neural regeneration and have an overall anti-aging effect (Yasuhara et al. 2008, Tsivgoulis et al. 2015, Garatachea et al. 2015, Gioscia-Ryan et al. 2016). Future studies will serve to clarify the effects of NT-020 on the pathways discussed and further elucidate the compound’s beneficial effects.
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APPENDICES
Appendix A – The Role of Glycogen Synthase Kinase-3 Signaling in Neurodevelopment and Fragile X Syndrome
Introduction

Fragile X syndrome (FXS), originally known as Martin-Bell syndrome [1] was first characterized in 1943 [2] is the most common cause of inherited mental retardation and is the first identified autism-related genetic disorder. The primary symptom of FXS is intellectual disability, but patients also share characteristics commonly associated with autism spectrum disorders (ASDs), such as developmental delays, communication impairments, and anxiety [3-9]. The most severely affected FXS individuals additionally display dysmorphic features, and other neurological pathology, including seizures. In this review, genetic and molecular features of FXS are detailed in the context of FXS neuropathology. Finally, potential mechanisms by which FMRP silencing impacts GSK3 and GSK3-associated signaling pathways are discussed. As GSK3 signaling represents a central regulatory node for critical neurodevelopmental pathways, understanding how FXS results from FMRP-mediated GSK3 dysregulation may provide novel therapeutic targets for disease-modifying interventions for FXS and related ASDs.

Etiology of FXS

The first evidence regarding the molecular origin of FXS was generated in 1969, at which time a non-typical constriction, or fragile site, was observed at the end of the X chromosome in several affected individuals [10, 11]. In 1991, the fragile site was mapped to a specific location in the genome [10]. The fragile X mental retardation 1 (Fmr1) gene on the X chromosome was found to yield a lack of the gene product, the fragile X mental retardation protein (FMRP), an RNA binding protein which regulates translation [3, 12]. This functional loss typically occurs when there is an expansion of the CGG trinucleotide repeat in the 5′ untranslated region (5′
Glycogen synthase kinase-3 and fragile X syndrome

UTR) of the fragile X mental retardation 1 (FMR1) gene [13]. Healthy individuals usually have between 5 to 54 repeats, but fully affected individuals have greater than 200 CGG repeats on what are known as “full mutation alleles” [14]. Permutation alleles (55-200 CGG repeats) of the FMR1 gene contribute to the FXS phenotype through genetic instability and can expand into the full mutation during the process of germline transmission [15, 16].

FMRP contains three RNA-binding domains and binds to a significant number of mRNAs. In in vitro studies, it has been found that dihydroxyphenylglycine-activated protein synthesis in synaptoneurosomes is reduced in a mouse model of FXS (the Fmr1 knockout mouse), which cannot produce full-length FMRP, suggesting that FMRP is involved in this process. FMRP is generated in synaptoneurosomes in response glutamate or metabotropic glutamate receptor (mGluR) agonists. Moreover, Fmr1 knockout mice demonstrate a substantial reduction in the ability to translate mRNA in response to activation in an experimental synaptoneurosome preparation as well as a reduction in the presence of postsynaptic polyribosomal aggregates in vivo [17].

Neuronal morphology and function in FXS

FXS patients and murine models of FXS demonstrate increased long-term depression (LTD) in hippocampal synapses [18]. FMRP functions to inhibit the synthesis of proteins that stabilize LTD. With functional loss of this protein, metabotropic glutamate receptor-5 (mGluR5) remains active and increases the synthesis of proteins associated with LTD. Increased activation of mGluR5 (and consequent increase in glutamate activity) has been implicated in audiogenic seizure activity associated with FXS. A study utilizing an mGluR antagonist and lithium to treat Fmr1 knockout mice found that the treatment alleviated mGluR-induced LTD [18]. Visualization of dendrites and dendritic spines can be performed using Golgi staining, which allows for quantitative evaluation [19] of developmental pruning of neural processes [20-22]. In humans, spine density on the dendritic apical shafts of cortical pyramidal cells increases within the first few months of life [23]. Autopsy tissue of normal human subjects ranging in age from fetal to adult revealed synapse density peaks between 3 months and 3.5 years, depending on the cortical region in question [24, 25]. Following this initial burst of synaptic development, synapses are selectively pruned, leaving synapse density measures at approximately 60% of their original peak numbers [26, 27], although somewhat smaller losses are observed when neuron density is taken into account [25]. Regardless of the biological basis for this developmental delay, dendritic spine dysgenesis frequently characterizes neuronal morphology in disorders associated with intellectual disability [28]. Studies utilizing samples from patients with FXS have suggested that dendritic spines do not assume a normal mature size and shape and that there are more dendritic spines per unit dendrite length in the patient samples compared to unaffected individuals. Similar findings on spine size and shape have come from studies of FXS model mice in which the development of the somatosensory cortical region contains barrel-like cell arrangements that process whisker sensory information [29]. This suggests that normal dendritic pruning is impaired in the knockout mice [17] and indicates that FMRP may be required for the normal processes of maturation and elimination to occur in cerebral cortical development [17].

Structural magnetic resonance imaging (MRI) has shown a reduction in the size of the posterior cerebellar vermis which may result in the enlargement of the fourth ventricle in males with FXS [30, 31]. Such gross morphological aberrations are not unique to the cerebellum, because the volume of hippocampus [32], caudate nucleus, and lateral ventricles [33, 34] also have all been noted to be enlarged in FXS patients. The generalizability of these observations is controversial as several of these differences in brain morphology have not been replicated in a study using physical measurements of autopsy material from one underpowered study (2 FXS patients) [35].

At the molecular level, the consequence of the aforementioned CGG trinucleotide expansion in the 5’ untranslated region of the FMR1 gene leads to a hypermethylation of the promoter region of the DNA, thus silencing transcription of the gene and resulting in the absence of FMRP. The function of FMRP has not yet been fully elucidated, although it is found to be associated with polyribosomal complexes near synapses and contains mRNA-binding domains. This suggests that it may be involved in mRNA
transport or translation of proteins required for synaptic plasticity [36]. FMRP’s role as an mRNA-binding protein is so critical for normal development that a point mutation in one of its RNA binding sites results in severe intellectual disability [37].

To test indirectly the role of FMRP in neuroplasticity, FMRP expression has been analyzed in rats after exposure to experimental paradigms known to induce synaptic plasticity. Regional increases in FMRP immunoreactivity were observed after training on a motor learning task or exposure to a complex environment [38]. It has also been shown that cortical levels of FMRP are elevated following sensory stimulation [39]. These observations suggest that the expression of FMRP is activity-dependent, and that the protein is involved in processes underlying synaptic plasticity [40]. Thus, it has been suggested that the loss of FMRP may lead to deficits in synaptic plasticity that could impair neuronal development [17].

Several other studies of FXS patients support that the syndrome is associated with dendritic spine dysgenesis, suggestive of abnormal neuronal development. In qualitative studies of Golgi-impregnated cortical neurons from human autopsy tissue, immature-appearing dendritic spine morphology has been described [41, 42]. Specifically, long, thin, tortuous spines with prominent heads and irregular dilations on apical dendrites of pyramidal cells in layers III and V of parieto-occipital neocortex and in the pyramidal layer of allocortex have been observed. Investigators noted that this spine morphology was reminiscent of that described in children and infants with other disorders associated with intellectual disability, such as Down syndrome and Patau syndrome [43]. Decreased synaptic contact area also was found, but no other major neuropathologies were observed. The lack of altered neuronal density in FXS patients, with the absence of significant cortical atrophy in the MRI research, indicates normal neurogenesis and cell migration and no prominent atrophy of processes.

The role of GSK-3 in neurodevelopment

Glycogen synthase kinase-3 (GSK-3) regulates a variety of developmental processes, such as neurogenesis, gliogenesis, cell migration, cell morphology, and axonogenesis through interaction with a variety of signaling pathways [44-47]. GSK-3 is a partially constitutively active serine/threonine kinase that is predominantly modulated by inhibitory serine phosphorylation of its two isoforms, serine-9 on GSK-3β and serine-21 on GSK-3α [48-50].

FMRP is known to play a critical role in adult hippocampal neurogenesis and regulates adult neural stem cell (NSC) fate by modulating the translation of glycogen synthase kinase-β (GSK-3β) [51]. One study examined the effects of GSK-3β inhibition on Fmr1 knockout mice [51]. GSK-3β inhibition increased hippocampal neurogenesis and improved performance in hippocampal-dependent learning tasks. It is possible that while overall neuronal density is not significantly altered in FXS patients, a decrease in hippocampal neurogenesis through loss of FMRP and the resultant dysregulation of GSK3 contributes to the pathogenesis of the disorder.

Underlying this are changes in the inhibitory serine-phosphorylation, which has a robust impact on GSK-3 activity, as this is the cardinal mechanism by which it is regulated. The phosphoinositide-3-OH kinase (PI3K)/Akt pathway is an essential pathway for neuronal and glial survival and is also one of the main regulatory pathways for serine-phosphorylation of GSK3β and GSK-3α [47, 52]. However, GSK-3β can also be regulated by p38 mitogen-activated protein kinase (MAPK)-mediated inhibitory phosphorylation of serine-389 [53]. While GSK-3β and GSK-3α are expressed ubiquitously, GSK-3β2 is highly expressed in the central nervous system (CNS) and is found in highest concentrations during neurodevelopment [44].

GSK-3 inactivation has been associated with increased neuronal progenitor proliferation and suppressed neural differentiation. GSK-3 interacts with the canonical Wnt, sonic hedgehog (SHH), and Notch pathways to regulate proliferation [22, 24, 43]. The canonical Wnt/β-catenin signaling pathway involves Wnt binding to Frizzled receptors (Fzd) and Fzd binding to the protein Disheveled (Dvl). Dvl then binds and destabilizes the β-catenin destruction complex, which includes GSK-3. Therefore, GSK-3 regulates the canonical Wnt pathway by remaining bound to the Wnt complex, preventing β-catenin from translocating to the nucleus to induce gene transcription [54]. Inhibition of GSK-3 is necessary for β-catenin-mediated transcription. Wnt/β
-catenin signaling is vital for adult hippocampal neurogenesis and is critical for CNS developmental processes, such as synapsete and dendrite formation.

**GSK-3 and fragile X syndrome**

GSK-3 activity has been found to be elevated in murine models of FXS [57]. A recent study found that lithium administration increased inhibitory phosphorylation of GSK-3 isoforms, reduced audiogenic seizure activity, and improved performance on open field, elevated plus maze, and passive avoidance tests in Fmr1 knockout (KO) mice, and passive avoidance tests [52]. Fmr1 KO mice also display impaired sociability. Mines et al. (2010) found that GSK-3 inhibition with lithium improved the previously impaired social interaction of Fmr1 KO mice with a novel mouse [58].

GSK-3 activity is also associated with mGluR5 in that mGluR5 normally activates the PI3K/Akt pathway, which induces inhibitory phosphorylation of GSK-3. However, it has been shown that mGluR5 signaling and GSK-3 activities are both elevated in Fmr1 KO mice [57]. A study utilizing both lithium and the mGluR inhibitor 2-methyl-6-phenylethynyl-pyridine (MPEP) found that the inhibition of mGluR also led to the inhibition of GSK-3. Both treatments led to decreased audiogenic seizure activity and improvement on open field tests. However, treatment with both lithium and MPEP did not have an additive effect, suggesting that the pharmacologic agents may target the same signaling pathway.

Likewise, long-term depression has been found to be increased in Fmr1 KO mice [60]. One study found that Fmr1 knockout mice displayed less fear memory in contextual fear conditioning than wild-type mice and decreased long-term potentiation (LTP) in the anterior cingulated cortex and lateral amygdala (areas important for associative learning) [61].

Another way in which GSK-3 may be culpable in the cognitive deficits and altered brain pathology observed in FXS is through regulation of glycogenolysis. GSK-3 inhibits glycogen synthase, thus reducing glycogenolysis. Inhibition of glycogenolysis produces learning and memory deficits. Thus, the increased GSK-3 activity observed in FXS patients and Fmr1 KO mice may cause intellectual disability through negative regulation of glycogenolysis in the CNS.

While it is evident that GSK-3 inhibition has a therapeutic effect in Fmr1 KO mice and that GSK-3 plays a role in neuronal morphology and proliferation, it is not clear whether and to what extent GSK-3 is responsible for Fragile X neuronal and brain pathogenesis. Further analysis is required to glean the role of GSK-3 in neurodevelopment in murine models of FXS (Figure 1).

**Potential therapy and future directions**

FXS is generally believed to be a neuronal disorder due to the aforementioned behavioral and cognitive deficits and abnormalities in neuron morphology. Neuronal function is modulated by an array of immune cells and there is convincing evidence of neuronal dysfunction resulting from neuroinflammation [41, 42]. Though a role for immune activation and associated inflammation in autism is controversial [62-64], there is evidence of activated glia in autism [65-68] and disregulated plasma cytokines associated with FXS [21]. Additionally, reactive astrocytes have been found in many brain regions of Fmr1 knockout mice. This pathology was attenuated with lithium treatment, providing further evidence of the involvement of GSK-3 in FXS [69]. Another study found that treatment of Fmr1 KO mice with minocycline (an antibiotic that exerts anti-inflammatory effects), improved dendritic spine formation and performance on behavioral tests [70].

It has been speculated that maternal immune activation (MIA) may play a role in the development of autism through activation of inflammatory pathways in utero [71]. MIA can negatively impact fetal brain development and may impair social behavior [72]. A study of MIA in normal mice revealed an increase in interleukin-6 (IL-6) [74]. IL-6 is known to induce phosphorylation of Janus kinase-2 (Jak2) and signal transducer and activator of transcription-3 (STAT3), leading to the release of proinflammatory cytokines, such as TNF-α and IL-1β. Treatment with the bioflavonoid diosmin reduced inflammation in the brain tissue of MIA offspring [72]. Another study found that GSK-3 and STAT3 enhance production of IL-6 after immune activation, GSK-3 was also found to be critical in the interferon-γ (IFN-γ)-induced activation of STAT3 [51]. Therefore, it is possible that quelling the inflammatory
environment through modulation of GSK-3 is a mechanism by which a therapeutic effect may be achieved in Fmr1 KO mice.

Another way to accomplish this may be to use bioflavonoids to inhibit GSK-3 activity. It has been shown that GSK-3β activity is decreased in pancreatic cancer cells when they are treated with various citrus flavonoids [73, 74]. The impetus for targeting GSK-3 in pancreatic cancer cells is that GSK-3β is over-expressed in the nucleus of these cells and causes nuclear factor -κB (NF-κB) to become active and induce an inflammatory cascade. Thus, attenuation of the inflammation leads to decreased cancer cell proliferation. Treatment with the bioflavonoid
luteolin has also been shown to reduce amyloid plaques in a transgenic (Tg2576) mouse model of Alzheimer’s disease through modulation of GSK-3α [75]. To date, bioflavonoid-induced inhibition of GSK-3 has not been studied in other CNS-related disorders.

As noted earlier, lithium has been shown to be beneficial for Fmr1 KO mice in reducing the occurrence and severity of audiogenic seizures and ameliorating behavior deficits. However, cessation of lithium treatment has led to the reemergence of the FXS phenotype in Fmr1 KO mice [76]. Thus, lithium would have to be chronically administered to patients for the duration of the lifespan. Unfortunately, lithium can be highly toxic and may not be feasible as a prophylactic or therapeutic agent for pregnant mothers or young children [18, 77]. Bioflavonoids and other anti-inflammatory agents that serve as GSK-3 inhibitors may prove to be more viable and safe therapeutic options in the future.

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Glycogen synthase kinase-3 and fragile X syndrome


Appendix C – Potential Autoepitope within the Extracellular Region of Contactin-Associated Protein-like 2 Mice
Potential Autoepitope within the Extracellular Region of Contactin-Associated Protein-like 2 in Mice

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Abstract

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Authors' contributions
This work was carried out in collaboration between all authors. Author DFO designed and implemented the bioinformatics methods, assisted with the design of other experiments, analyzed the data and composed the manuscript. Authors YZ, ARB, and HH assisted in bioinformatics methods, performed experiments and assisted in analysis of data, manuscript composition, and editing. Authors SMP, JZ, SLS, TKM, and MAB assisted in data analysis, manuscript composition and editing. Author JT supervised and initiated the studies, performed analysis of data and assisted in the composition of the manuscript. All authors except author YZ read and approved the final manuscript.

CONSENT
Informed consent was obtained from the parents or guardians of all subjects by AGRE.

ETHICAL APPROVAL
All experiments involving human subjects have been examined and approved by institutional review boards of the University of South Florida, Morsani College of Medicine and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. AGRE has IRB approval from Western Institutional Review Board, Inc. and UCLA. Regarding experiments involving non-human animals “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985) were followed, and all experiments and tissue collection were conducted in accordance with the institutional guidelines and were approved by the University of South Florida Institutional Animal Care and Use Committee.

COMPETING INTERESTS
JT holds the Silver Chair in Developmental Neurobiology. TKM holds the Rothman Chair in Developmental Pediatrics and has received research support from NIHC in the past 3 years, International OCD Foundation, Tourette Syndrome Association, CDC, NARSAD, All Children’s Hospital Research Foundation Shire, Transcept Pharmaceuticals, Inc., Forest Laboratories, Janssen Pharmaceuticals and Otsuka Pharmaceuticals. TKM is on the Medical Advisory Board for Tourette Syndrome Association and Scientific Advisory Board for IOCDF. She receives textbook honorarium from Lawrence Erbbaum and research support from the Maurice and Thelma Rothman Endowed Chair. The other authors report no other biomedical financial interests or potential conflicts of interest. No writing assistance was utilized in the production of this manuscript.
Aims—Implicated in autoimmune encephalitis, neuromyotonia and genetic forms of autism, here we report that contactin-associated protein-like 2 (CNTNAP2) contains a potential autoepitope within the extracellular region.

Methodology—CNTNAP2 sequence-similar regions (CSSRs) from human pathogens were identified. Sera from autistic and control children were obtained and analyzed for the presence of antibodies able to bind CSSRs. One such candidate CSSR was evaluated for evidence of autoimmune responses to CNTNAP2 in a mouse model of acute infection.

Results—Autistic and control children sera contained antibodies able to discrete regions of CNTNAP2. In a murine model of acute infection, a CSSR derived from the N-terminal extracellular region of CNTNAP2 resulted in anti-CNTNAP2 antibody production, proinflammatory cytokine elevation, cerebellar and cortical white matter T-cell infiltration as well as motor dysfunction.

Conclusion—Taken together, these data suggest that CNTNAP2 contains a potential autoepitope within the extracellular region.

Keywords
CNTNAP2; Caspr 2; autoantibody; molecular mimicry; autoimmune; autoepitope; autism; encephalopathy

1. INTRODUCTION
Molecular mimicry is a process whereby an amino acid sequence-similar region, or shape-similar region, of a protein or other non-protein compound from an offending pathogen or other agent induces the production of adaptive immune system elements such as antibodies and/or T-cell receptors capable of cross-reacting with proteins or other compounds of the host. This pathological mechanism is thought to be involved in several conditions including: paraneoplastic cerebellar syndrome [1], autoimmune neuromyotonia [2], multiple sclerosis and related disorders [3,4], NMDAR encephalitis, and other autoimmune encephalopathies [5–7] as well as some developmental disorders including autism spectrum disorders [8–12]. Interestingly contactin-associated protein-like 2 (CNTNAP2), also known as Caspr2, is a member of the neurexin family and the target of autoantibodies thought to result in autoimmune encephalitis and neuromyotonia [2,13]. Furthermore, CNTNAP2 gene mutations are associated with autism [14–16]. The CNTNAP2 protein is expressed on neurons, neural stem cells, and astrocytes and is known to function in potassium channel clustering on myelinated axons, neuronal migration, membrane excitability, and neuron-glial interactions [17–19]. In early childhood, inflammatory insults may alter brain development as significant cross-over exists between molecular signaling pathways critical for brain development and those involved in immune responses [20–23]. Given its involvement in autoantibody-mediated neuroinflammatory disease, the investigations presented here evaluated CNTNAP2 for potential autoepitopes through a bioinformatics approach coupled with characterization of human CNTNAP2 binding antibodies from autistic and non-autistic children, and evaluation of a pathogen protein with similar linear protein sequences to human CNTNAP2 in a mouse model of acute infection.
2. METHODOLOGY

2.1 Cell Culture

Murine neuroblastoma cells (N2a) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in Dulbecco’s minimum essential medium (DMEM, Life Technologies, Gaithersburg, MD) with 100 μg/mL penicillin and streptomycin antibiotics at 5% CO₂ and 37°C. N2A cells were induced to differentiate by addition of 0.3 mM dibuturyl cAMP for 48–72 h. Cells were cultured in 24 or 96 well tissue culture for experimentation.

Mouse primary culture neuronal cells were isolated between E16 and E18 from cerebral cortices of mice subjected to LPS pre-treatment and CSSR3 peptide immunization. Cortices were incubated for 15 min in 0.25% trypsin at 37°C, and then mechanically dissociated. Single cells were collected after centrifugation at 290 x g and resuspended in DMEM supplemented with 10% fetal calf serum, 10% horse serum, uridine (33.4 μg/ml; Sigma) and fluorodeoxyuridine (13.6 μg/ml; Sigma). Cells were then plated in collagen-coated 24-well tissue culture plates at 2.5 × 10⁵ cells per well for experimentation.

2.2 Enzyme-Linked Immunosorbent Assays

CNTNAP2 sequence-similar regions (CSSR) were determined comparing human [NCBI Reference Sequence: NP_054860.1] and mouse [NCBI Reference Sequence: NP_001004357.2] CNTNAP2 proteins [24] against non-redundant protein sequences from available bacterial and viral protein databases using the Protein-Basic Local Alignment Search Tool (BLAST) [25] (Step 1). Resulting CSSRs (Table 1) at least 5 amino acids (aa) in length, within the predicted extracellular region of both human and murine CNTNAP2, were further analyzed by a battery of B-cell epitope prediction software tools [26–28] (Step 2). From those, only known human pathogens (bacterial or viral only) proteins were selected and then further analyzed based on linear protein sequence similarity with known B-cell epitopes [29] (Step 3). The final CSSRs from Step 3 were selected a priori and synthetic peptides (Table 2) were designed to contain a given CSSR flanked by sufficient amino-acids from human CNTNAP2 so as to generate peptides 8 amino acids in length.

A region of human CNTNAP2 at amino acids (aa) 41–49, not containing sequence similarity with the final CSSR peptides, was selected as a peptide control. Antibody titers were quantified using ELISA whereby individual CSSR peptides were first diluted to 1 μg/mL in 50 mM carbonate buffer (pH 9.6) and then used to coat 96-well plates at 100μL per well for 18 h at 4°C. Plates were next washed 5 times with phosphate buffered saline (PBS), 0.05% TWEEN-20, at pH 7.4 (wash buffer). Wells were blocked with 1% bovine serum albumin (BSA) and 5% horse serum in PBS for 2 h at room temperature. Following blocking, the plates were washed 5 times with wash buffer. Sera samples from autistic and control children (Table 3) were diluted (1:100) with 1% BSA in PBS.

Samples and standards were incubated in plate for 2 h at room temperature. After this incubation, the plates were washed 5 times with wash buffer, secondary antibody (anti-human IgG conjugated with HRP, produced in rabbit, 1:5000 dilution) incubation was conducted for 2 h at room temperature then 5 further washes with wash buffer and finally
the plates were developed with tetramethylbenzidine substrate-chromogen (Dako, Carpinteria, CA, USA). The reaction was stopped with 2 N sulfuric acid and the plates were analyzed spectrophotometrically at 450 nm. Commercially available ELISA kits were used to measure tumor necrosis factor-α (TNFα; eBioscience, San Diego, CA) and interferon-γ (IFNγ; R&D Systems, Minneapolis, MN) levels in mouse sera and brain tissues. Experiments were performed according to manufacturers’ instructions.

2.3 Cytotoxicity Assay

Sera from individual mice were pooled together based on treatment group after isolation. Next 10 μL was diluted (1:100) in culture media and then incubated with N2a cells in 96 well plate for 24 h with and without 1 hour pre-incubation with CSSR3 or CNTNAP2 ctrl peptides (5 μg/mL). Media were then collected and analyzed for lactate dehydrogenase (LDH) release (Sigma) according to the manufacturer’s instructions.

2.4 Mouse Husbandry and Treatment

Wild-type C57BL/6 mice were purchased (Jackson Laboratories, Bar Harbor, ME) and housed in a 12-h light-dark cycle. Mice (4 week old, n = 8, 4♀/4♂ per group, 6 groups, total 54 mice) were treated via intraperitoneal (i.p.) injection with PBS or LPS (10 μg/mouse); and with and without immunization against (200 μg/mouse) synthetic peptides including pathogen peptide (NCBI Reference Sequence: NP_880571.1, filamentous hemagglutinin protein from Bordetella pertussis, 3034–3053 aa, sequence AGTSVDA\textit{ANVSID}AGKDLNL) containing CSSR or Control peptide (NCBI Reference Sequence: NP_054860.1, CNTNAP2 31–50 aa, sequence TSQKCDEPLVSGLPHVAFSS); a portion of CNTNAP2 found not to have significant linear protein sequence similarity to known human bacterial or viral pathogen proteins. All treatments were repeated after one week.

For tissue collection, mice were anesthetized using gaseous isoflurane. Blood was collected from the right ventricle of the heart and immediately placed into tubes containing 0.5 M EDTA (BD Biosciences, San Jose, CA). Mice were transcardially perfused with cold 0.01 M PBS (pH 7.4) and brains were rapidly removed and sagittally bisected. Left hemispheres were separated into cerebrum and cerebellum regions before homogenization in 1 X lysis buffer (Cell Signaling, Boston, MA) with 1% PMSF (Sigma), centrifuged at 14,000 rpm for 15 min and stored at −80°C. Right hemispheres were fixed overnight with 4% paraformaldehyde and cryoprotected in a graded series of 10%, 20% and 30% sucrose solutions, each overnight at 4°C. Right hemispheres were then embedded in Neg50 frozen section medium (Richard-Allan Scientific, Kalamazoo, MI), and coronally sectioned on a Microm HM 550 cryostat (Thermo Scientific, Richard-Allan Scientific, Kalamazoo, MI) at 25 μm thickness. Free-floating sections were preserved in PBS containing 100 mM sodium azide at 4°C. All experiments and tissue collection were conducted in accordance with the institutional guidelines and were approved by the University of South Florida Institutional Animal Care and Use Committee.
2.5 Immunohistochemistry

Mouse brain tissue sections were washed in PBS, blocked in 5% horse serum/PBS for 1 h at room temperature and incubated with primary antibodies against CD3 or CD4 (rat, 1:1,000) overnight at 4°C in blocking solution. Sections were then washed and incubated for 1 h with biotinylated secondary antibody (anti-rat, 1:200) that was viewed by the ABC kit (Vector Laboratories) with diaminobenzidine (DAB). Slides were counterstained with hematoxylin. Images were obtained using an Olympus BX-51 microscope.

2.6 Motor Function Analysis

Motor coordination and balance were tested by placing each mouse on a rotating drum (RotaRod, UgoBasile, Stoelting, Wood Dale, IL) stationary and during acceleration from 0 to 40 rpm over a 5 minute period. Six mice per trial were randomly evaluated by a technician blinded to their identities. Each mouse was subjected to this task 5 times with a 30-min interval between each trial on RotaRod. All mice were tested on the same day.

2.7 Human Sera Samples

Sera from autistic (n = 26) and non-autistic children (n = 18) aged 3–11 years (Table 3) were obtained from the Autism Genetic Research Exchange (AGRE) [30]. Approval for study involving these specimens was granted by the institutional review boards of the University of South Florida, Morsani College of Medicine and AGRE. Autism diagnosis was determined using the Autism Diagnostic Interview - Revised (ADI-R) [31].

2.8 Statistical Analyses

All data were normally distributed; therefore, in instances of single mean comparisons, Levene’s test for equality of variances followed by t-test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance (ANOVA) was used, followed by post-hoc comparison using Bonferonni’s method. Alpha levels were set at .05 for all analyses. The statistical package for the social sciences release 18 (SPSS, Chicago, IL) was used for all data analysis.

3. RESULTS AND DISCUSSION

3.1 CSSR of Proteins from Human Pathogens

Molecular mimicry is a well-known phenomenon known to underpin many disorders. To test the hypothesis that linear protein sequences from known human viral and bacterial pathogens could be useful in predicting potential autoepitopes on human CNTNAP2 we screened for CSSRs by comparison of human and mouse CNTNAP2 proteins against known bacterial and viral protein databases using NCBI Protein-BLAST [25] (Table 1). The candidate CSSR had to be at least 5 aa in length, within the predicted extracellular region of both human and murine CNTNAP2, be within a predicted B-cell epitope [26–28], and be from a known human pathogen. The final 8 aa peptides containing CSSRs were lastly selected a priori for further evaluation (Table 2).
3.2 CNTNAP2-binding Antibodies in Sera from Children with Autism and Non-autistic Controls

Sera from children 3–11 years of age with autistic disorder (n = 26), and non-autistic controls (n = 18), were obtained (Table 3) and screened by ELISA for the presence of antibodies against 8 aa peptide targets of CNTNAP2 (Table 2) containing sequence-similarity with proteins from known human pathogens. Compared with the CNTNAP2 control peptide target, significant elevations in antibody binding were only observed to CSSR3 and CSSR4 in those with autism (Fig. 1). Although pathogen exposure profiles of the individuals are unknown and the groups are characteristically dissimilar (Table 3) these observations suggested that some children have circulating antibodies able to bind regions of CNTNAP2 that are sequence-similar to proteins from known human pathogens.

3.3 CNTNAP2 Binding Antibodies Generated in Mice Pre-injected with LPS and Immunized with a Pathogen Peptide Containing the CSSR

Next, given that some children displayed elevations in serum antibody binding to its target sequence CSSR3 was selected for functional characterization in a mouse model of acute infection. Four-week-old mice C57BL/6 mice were subjected to PBS or LPS pre-treatment (10 μg/mouse) 2 days prior to immunization with a 20 aa peptide from pathogen peptide containing the CSSR (PPC) or control peptide (a portion of CNTNAP2 found not to have significant linear protein sequence similarity to known human bacterial or viral pathogen proteins). The same procedure was repeated one week later and mice were sacrificed after motor function testing; at approximately 8 weeks. Only those mice treated with both LPS pre-treatment and PPC expressed significantly elevated levels of antibodies able to bind the CSSR3 peptide (Fig. 2B) by ELISA. This suggested that in mice a peptide derived from a pathogen protein with a CSSR could induce the generation of antibodies binding the analogous region of CNTNAP2 with LPS pretreatment. As expected, LPS pre-treatment was associated with serum TNFα elevations (Fig. 2A).

3.4 CSSR3 Peptide Binding Antibodies Injure Neuronal Cells

To further characterize the functional effects of CSSR3 binding, the pooled sera from the same mice were further analyzed through incubation with neuronal cells to monitor cell death by LDH release over 24 h. Sera from the PPC treated group pre-treated with LPS, but not sera from the group treated with PPC alone or control peptide with or without LPS-pre-treatment, displayed significant elevations in LDH release by differentiated neuron-like N2a cells (Fig. 3A) and murine primary culture neurons (Fig. 3B).

The LDH release of the sera from mice subjected to PPC immunization and LPS pre-treatment could be mitigated by pre-mixing the sera, prior to incubation with neuronal cells, with the CSSR3 peptide but not the CNTNAP2 ctrl peptide (Fig. 3C). These observations suggested that CSSR3 binding antibodies produced in mice pre-treated with LPS displayed neurotoxic properties dependent on their ability to bind a specific extracellular region of murine CNTNAP2 (545–550 aa).
3.5 Elevations in CD3+ Cells in Brains of Mice Treated with LPS and PPC

Several brain regions were evaluated by immunohistochemistry (IHC) for CD3+ immunoreactivity. Although no differences were observed between the groups in the analyzed regions of cortical gray matter (Fig. 4A), mice subjected to LPS pre-treatment and immunization with PPC displayed increased cortical white matter as well as cerebellar CD3+ (Fig. 4B) and CD4+ immunoreactivity (data not shown); whereas other groups displayed predominately blood vessel associated T-cell CD3+ and CD4+ immunoreactivity.

3.6 IFNγ and TNFα Elevations in Brains of Mice Treated with LPS and PPC

Furthermore, dramatic elevations in central nervous system (CNS) levels of IFNγ (Fig. 5A) and TNFα (Fig. 5B) cytokines were observed in the LPS pre-treatment and PPC group compared with the LPS pre-treatment/control peptide group.

3.7 LPS and PPC Treatment Results in Abnormal Motor Function in Mice

Observations of elevations in CNTNAP2 autoantibodies have been made in some patients [13]; although often associated with muscular hyperactivity, including spasm, rigidity, and myotonia, fatigue and exercise intolerance are also frequently observed. Thus, the same C57BL/6 mice, 3 weeks after last treatment, prior to sacrifice, were evaluated for fatigue and exercise intolerance by RotoRod analysis. Mice in the LPS pre-treatment, PPC immunization group displayed significantly greater fatigue and exercise intolerance during RotoRod analysis including shorter times before falling from a stationary RotoRod (Fig. 6A) and shorter times before falling from an accelerating RotoRod (Fig. 6B) compared with other groups evaluated.

Autoantibodies observed in cases of autoimmune encephalitis and/or neuromyotonia have not been shown to involve binding to the region of CNTNAP2 characterized here (545–550 aa) and to our knowledge this is the first study to screen for potential autoantigenic regions of CNTNAP2 using the methods presented here. Known to be associated with immune system dysregulation [8–11,22,32–34], subsets of children with autism tend to display elevations of serum antibody binding to CSSR3 and CSSR4 (Fig. 1) compared with non-autistic children; however these data are hampered by the dissimilarity of non-CNTNAP2 binding antibody variables between the two groups evaluated (Table 3) and the unknown or incomplete medical histories of the patients.

Despite the relatively small region of similarity the 6 aa long CSSR of the PPC fulfills known requirements for antigen function [35–37]. The PPC was used to immunize mice pre-treated with LPS to further characterize the autoantigenic potential of a small segment (545–550 aa) of the extracellular region of human and murine CNTNAP2. This treatment was associated with elevations in antibodies able to bind the analogous region of CNTNAP2 (Fig. 2), CD3+ and CD4+ (data not shown) cells and inflammatory cytokines in CNS tissues (Fig. 4, 5) and motor dysfunction (Fig. 6); only in the presence of LPS pre-treatment. LPS and PPC treated mice showed signs of an encephalitis-like reaction with increased parenchymal cortical white matter as well as cerebellar CD3+ (Fig. 4B) and CD4+ immunoreactivity (data not shown); whereas without the combination of LPS and PPC the pattern was that of blood vessel associated T-cell CD3+ and CD4+ immunoreactivity;
similar to untreated controls. These data together suggest that only in the presence of strong inflammatory responses, mimicking acute gram-negative bacterial infection driven by LPS, was immune tolerance to the CNTNAP2 self-antigen able to be broken.

Whereas in 4–8 week old mice treatment with LPS and PPC lead to encephalitis-like responses and motor dysfunction, it is tempting to speculate whether earlier exposure, perhaps in utero, would lead to more profound immune and nervous system dysfunction and abnormal development. In light of the key role of CNTNAP2 in neuronal activation, migration and neural-glial interactions [18,19,38] further studies are needed to determine whether anti-CNTNAP2-mediated encephalopathy, if occurring during critical widows of brain development, could represent a significant risk factor for abnormal neurodevelopment. Prior studies present compelling evidence of a role for the immune system in the pathogenesis of subsets of neurodevelopmental disorders [8–12,22,23,32–34].

Importantly, the results have significant limitations including that a synthetic linear peptide representing a small fragment of CNTNAP2, not in its native form, was used to immunize and evaluate the effects of the CSSR in mice and detect CSSR-binding antibodies in human samples. Although the antibodies binding to CSSR3 generated by LPS and PPC pretreatment appeared to bind CNTNAP2 in its native form on neuronal cells (Fig. 3), it remains to be determined whether the extracellular region of (545–550aa) CNTNAP2 analogous to the PPC would be available for antibody binding in its native conformation in humans. Further, the human sample data contained within the present study is significantly limited by the small sample size and dissimilarity between groups as well as the lack of or incomplete medical histories. Further complete immunological characterization of the evaluated mice was not completed prior or after LPS and PPC treatment.

4. CONCLUSION

CNTNAP2 contains a potential autoepitope within the extracellular region.

5. FUTURE DIRECTIONS

Molecular mimicry is implicated in neurological disorders associated with anti-CNTNAP2 antibodies. Analysis of the relative affinity of antibodies from patients with autistic disorder binding to the 545–550 aa region of CNTNAP2 against antisera from mice immunized using the same region as well as analysis of antibody affinity-to-neurotoxicity relationships and visualized regional binding characteristics on human neurons could support the hypotheses that antibodies binding to the 545–550 aa region of CNTNAP2 are causative in human neurological disorders.

Acknowledgments

This work is dedicated to the memory of Dr. Yuyan Zhu. The investigations presented here were supported by the Silver Endowment, Rothman Endowment and the NIH/NIMH (R21MH087849, JT). We gratefully acknowledge the resources provided by the Autism Genetic Resource Exchange (AGRE) Consortium* and the participating AGRE families. The Autism Genetic Resource Exchange is a program of Autism Speaks and is supported, in part, by grant 1U24MH081810 from the National Institute of Mental Health to Clara M. Lajonchere (PI). We thank the reviewers for their efforts and thoughtful comments.

Br J Med Med Res. Author manuscript; available in PMC 2014 January 22.
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADI-R</td>
<td>Autism Diagnostic Interview, Revised</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BLAST</td>
<td>The Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>Caspr2</td>
<td>contactin-associated protein-like 2</td>
</tr>
<tr>
<td>CD3</td>
<td>cluster of differentiation 3</td>
</tr>
<tr>
<td>CD4</td>
<td>cluster of differentiation 4</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTNAP2</td>
<td>contactin-associated protein-like 2</td>
</tr>
<tr>
<td>CSSR</td>
<td>CNTNAP2 sequence similar region</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>i.p</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LDH</td>
<td>lactose dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>molarity</td>
</tr>
<tr>
<td>N</td>
<td>Normality</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPC</td>
<td>pathogen peptide containing CSSR</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor-alpha</td>
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</table>

References


Fig. 1.
CNTNAP2-binding antibodies in sera from children with autism and non-autistic controls. Levels of serum antibodies binding to 8 aa CNTNAP2 autoantibody detection peptides containing analogous CSSR sequence from corresponding pathogen proteins (Table 2) were screened by ELISA. Each dot represents a mean optical density reading (O. D.; 450 nm; 1:100 dilution) for each individual (n = 26 for autistic children; n = 18 for non-autistic controls) for a respective level of serum antibodies binding to a given CNTNAP2 autoantibody detection peptide (CR). Levels of CSSR3 (CR3) and CSSR4 (CR4) autoantibody titers were significantly elevated in children with autism compared with non-autistic control sera (P < .05). There were no significant differences in autoantibody titer binding to other CNTNAP2 autoantibody detection peptides compared to CNTNAP2 control peptide (P > .05)
Fig. 2.
CNTNAP2 binding antibodies generated in mice pre-injected with LPS and immunized with PPC. Wild-type C56BL/6 (WT) mice (4 week old, n = 8, 4♀/4♂ per group) intraperitoneally (i.p.) injected with PBS or LPS (10 μg/mouse) with and without control peptide (Ctrl) or PPC (200 μg/mouse) immunizations. The same procedure was repeated one week later.
ELISA for TNFα (A) and CSSR3 binding antibody titer (B) were determined 3 weeks after last immunization. The results are presented as mean ± SD of TNFα (pg/mL) for (A) and mean ± SD of O.D. reading at 1:100 dilution for (B). ***P <.001
Fig. 3. CSSR3 peptide binding antibodies injure neuronal cells. Pooled sera from control and PPC immunized groups were collected 3 weeks after last immunization (8 week old, n = 8, 4♀/4♂ per group) and used to treat differentiated N2a cells (A) and mouse primary neuronal cells (B) for 24 h and analyzed by LDH assay. Data are presented as mean ± SD of LDH release (%) from each incubated group normalized by total cellular protein. (C) Mouse primary neuronal cells incubated with sera from “control mice” (immunized with Ctrl peptide + LPS pre-injection) or “PPC mice” (immunized with PPC + LPS pre-injection) for 24 hours with and without pre-incubation with CSSR3 or CNTNAP2 ctrl peptides (5 μg/mL) for 1 h at 37°C. Ctrl sera, the pooled serum from “control mice.” Ctrl sera/CSSR3, pooled serum from “control mice” pre-incubated with CSSR3 peptide. Ctrl sera/CNTNAP2 ctrl, pooled serum from “control mice” pre-incubated with CNTNAP2 ctrl peptide. Sera, the pooled serum from the PPC mice. Sera/CSSR3, the pooled serum from the PPC mice pre-incubated with CSSR3 peptide. Sera/CNTNAP2 ctrl, the pooled serum from the PPC mice pre-incubated with CNTNAP2 ctrl peptide.
Fig. 4.
Elevations in CD3+ cells in brains of mice treated with LPS and PPC. (A) Representative gray matter cortical regions from mice immunized with control peptide (Ctrl) or PPC peptide with pre-treatment with LPS (8 week old, n = 8, 4♀/4♂ per group). Significant CD3+ T-cell infiltration was not observed in the areas of cortical gray matter analyzed in either group. (B) Representative cortical cerebellar and cortical white matter regions from mice immunized with Ctrl peptide or PPC with LPS pre-treatment. Mice that received LPS pre-injection and were immunized with PPC peptide displayed CD3+ T-cell infiltration in the cerebellum and cortical white matter. Mice immunized with Ctrl peptide + LPS pre-injection did not display CD3+ T-cell infiltration in these regions.
Fig. 5.
IFNγ and TNFα elevations in brains of mice treated with LPS and PPC. Mouse brain tissue homogenates (8 week old, n = 8, 4♀/4♂ per group) were evaluated for IFNγ (A) and TNFα (B) by ELISA. The results are presented as mean ± SD of brain IFNγ or TNFα (pg/mg total protein). Both cytokines were not detectable in brain tissues from other control groups (data not shown). *P < .05; **P < .005; ***P < .001
Fig. 6.
LPS and PPC treatment results in abnormal motor function in mice. Wild-type C56BL/6 mice were immunized with PPC or control peptide (Ctrl) with LPS or PBS pre-treatment and subjected motor function testing (8 week old, $n = 8$, 4♀/4♂ per group). Motor balance analysis was conducted using RotoRod. (A) Time before falling from a stationary RotoRod. (B) Time before falling off an accelerating RotoRod. Results are presented as mean ± SD from five trials. ***$P < .001$
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<thead>
<tr>
<th>Protein</th>
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<tr>
<td>[YP_00627301.1] ABC transporter, Bacillus thuringiensis</td>
<td>105 LKLDHYP 111</td>
<td>127 LKLDHYP 113</td>
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<td>[WP_006090500.1] amidase, Desulfovibrio vulgaris</td>
<td>138 PWFDH 142</td>
<td>1230 PWFDH 1234</td>
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<td>156 ARYVR 160</td>
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<td>170 PVFDH 174</td>
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<td>138 PWHLD 142</td>
<td>1230 PWHLD 1234</td>
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<td>[YP_005986680.1] beta-N-acetylhexosaminidase, Propionibacterium acnes</td>
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<td>220 LVFSH 224</td>
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<td>3041 ANVSR 3046</td>
<td>545 ANVSR 550</td>
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<td>[ABX58360.1] hemagglutinin, Influenza A virus</td>
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<td>335 GCMES 339</td>
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<td>127 LKLDHYP 113</td>
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<td>1068 SSFTT 1072</td>
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<td>1231 WHLDH 1235</td>
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<td>[WP_004614047.1] hypothetical protein, Clostridium bolteae</td>
<td>1308 ARYVR 1312</td>
<td>156 ARYVR 160</td>
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Peptide sequences are identified by National Center for Biotechnology Information (NCBI) or GenBank accession numbers.

*Not recognized as direct human pathogens.
Table 2

CNTNAP2 Autoantibody Detection Peptides

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<tr>
<th>Pathogen NCBI accession #, peptide name</th>
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<tr>
<td>[NP_880571.1], CSSR1</td>
<td>GEGRIGLR</td>
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<td>[AAL83746.1], CSSR5</td>
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<td>[O40955], CSSR6</td>
<td>DPWHLDHL</td>
<td>CNTNAP2-1231</td>
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<tr>
<td>[NP_054860.1], CNTNAP2 Control</td>
<td>SGLPHVAF</td>
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### Table 3

Characteristics of control and autistic children

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<tr>
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<th>Controls (n=18)</th>
<th>Autism (n=26)</th>
<th>p value</th>
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<tbody>
<tr>
<td>Female (%)</td>
<td>44.44</td>
<td>15.39</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Age (years)</td>
<td>5.71±1.42</td>
<td>4.00±1.366</td>
<td>&lt;.001</td>
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</table>
Appendix B – GFAP Expression and Social Deficits in Transgenic Mice Overexpressing Human sAPPα
GFAP Expression and Social Deficits in Transgenic Mice Overexpressing Human sAPPα

Antoinette R. Bailey,1,2 Huayan Hou,1,2 Min Song,1,2 Demian F. Obregon,1,2 Samantha Portis,1,2 Steven Barger,3 Doug Shytle,4 Saundra Stock,2 Takashi Mori,5,6 Paul G. Sanberg,4 Tanya Murphy,7 and Jun Tan1,2

Autistic individuals display impaired social interactions and language, and restricted, stereotyped behaviors. Elevated levels of secreted amyloid precursor protein-alpha (sAPPα), the product of α-secretase cleavage of APP, are found in the plasma of some individuals with autism. The sAPPα protein is neurotrophic and neuroprotective and recently showed a correlation to glial differentiation in human neural stem cells (NSCs) via the IL-6 pathway. Considering evidence of gliosis in postmortem autistic brains, we hypothesized that subsets of patients with autism would exhibit elevations in CNS sAPPα and mice generated to mimic this observation would display markers suggestive of gliosis and autism-like behavior. Elevations in sAPPα levels were observed in brains of autistic patients compared to controls. Transgenic mice engineered to overexpress human sAPPα (TgsAPPα mice) displayed hypoactivity, impaired sociability, increased brain glial fibrillary acidic protein (GFAP) expression, and altered Notch1 and IL-6 levels. NSCs isolated from TgsAPPα mice, and those derived from wild-type mice treated with sAPPα, displayed suppressed β-tubulin III and elevated GFAP expression. These results suggest that elevations in brain sAPPα levels are observed in subsets of individuals with autism and TgsAPPα mice display signs suggestive of gliosis and behavioral impairment.

Key words: sAPPα, astrogliosis, autism, behavior, IL-6, Notch

Introduction

Autism is a heterogeneous neurodevelopmental disorder characterized by impaired communication, social interaction, and restricted, repetitive behaviors and interests (APA, 2000; Steyaert and De la Marche, 2008). Patients with autism exhibit varied patterns of aberrant neuroanatomical and immunological features in addition to behavioral phenotypes (Bailey et al., 1998; Bauman and Kemper, 2005; Casanova et al., 2006; Amaral et al., 2008). Brain overgrowth followed by decelerated development is frequently observed in some autistic patients. This neuropathology is observed early in the developmental course and is thought to involve defects in the normal “pruning” of early neural network arbors. After proliferation and dendritic arbor formation, pruning and programmed cell death are highly regulated multifactorial processes dependent on ordered molecular and cellular interactions involving various players including neural stem cells (NSCs), astrocytes, and microglia.

Typically, glial cells are generated after neurogenesis in the CNS (Sofroniew and Vinters 2010). The full complement of neurons appears during the embryonic period while most gliogenesis occurs within the first month after birth (Jacobson, 2000; Steyaert and De la Marche, 2008). Considering evidence of gliosis in postmortem autistic brains, we hypothesized that subsets of patients with autism would exhibit elevations in CNS sAPPα and mice generated to mimic this observation would display markers suggestive of gliosis and autism-like behavior. Elevations in sAPPα levels were observed in brains of autistic patients compared to controls. Transgenic mice engineered to overexpress human sAPPα (TgsAPPα mice) displayed hypoactivity, impaired sociability, increased brain glial fibrillary acidic protein (GFAP) expression, and altered Notch1 and IL-6 levels. NSCs isolated from TgsAPPα mice, and those derived from wild-type mice treated with sAPPα, displayed suppressed β-tubulin III and elevated GFAP expression. These results suggest that elevations in brain sAPPα levels are observed in subsets of individuals with autism and TgsAPPα mice display signs suggestive of gliosis and behavioral impairment.

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Introduction

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secreted APP (sAPPa). Conversely, the nonamyloidogenic pathway produces AD pathology (Mattson, 1997; De Strooper and Annaert, 2000). Among other fragments, the amyloid-β peptides which constitute the cytotoxic plaques characteristic of Alzheimer's disease (AD) pathology (Marz et al., 1999; Taga and Fukuda, 2005), led to the upregulation of the astrocyte marker glial fibrillary acidic protein (GFAP) (Bonni et al., 1997; Deverman and Patterson, 2009; Kirsch et al., 2010).

The gp130 pathway cooperates with the Notch1 pathway, which also enhances NSC commitment to glial fate by suppressing neuronal differentiation (Bhattacharya et al., 2008; Sugaya, 2008; Rodriguez-Rivera et al., 2009). There is also evidence of crosstalk between the Notch1 and gp130 pathways as increased Hairy and enhancer of split (Hes), resulting from Notch1 activation, promotes phosphorylation of signal transducer and activator of transcription 3 (STAT3), a transcription factor for GFAP (Grandbarbe et al., 2003; Nagao et al., 2007).

Given that astrocytes are important for synaptic pruning during development, promotion of astrocytic differentiation and abnormal glial activation or gliosis appears sufficient to affect neurodevelopment (Oland and Tolbert, 2011; Stephan et al., 2012). Several studies give evidence for gliosis in different brain regions of autistic patients (Ahlsen et al., 1993; Sabaratnam, 2000; Laurence and Fatemi, 2005; Vargas et al., 2005). How abnormal cellular, inflammatory, and neurotropic signals align leading to excessive, aberrant dendritic connectivity via impaired pruning in autistic individuals is still unclear.

The amyloid precursor protein (APP) is a single-pass transmembrane glycoprotein consisting of 695–770 amino acids and existing in three main isoforms (Selkoe et al., 1988; Turner et al., 2003). APP is processed according to two separate pathways that produce different protein fragments. Protein processing by the amyloidogenic pathway produces, among other fragments, the amyloid-β peptides which constitute the cytotoxic plaques characteristic of Alzheimer's disease (AD) pathology (Mattson, 1997; De Strooper and Annaert, 2000). Conversely, the nonamyloidogenic pathway produces secreted APPα (sAPPα), the α-C-terminal fragment (α-CTF) and others (Mattson, 1997; De Strooper and Annaert, 2000). In addition to many reported physiological functions of holo-APP, the peptide fragments each have distinct roles in a variety of cellular processes occurring in the brain and other organs (Turner et al., 2003). Numerous reports of sAPPα potentiating neurite outgrowth, preventing neuronal death and aiding in NSC proliferation confirm its neurotrophic and neuroprotective properties (Furukawa and Mattson, 1998; Fu et al., 2002; Copanaki et al., 2010). Further, studies in isolated human NSCs suggest that sAPPα may promote astroglial cell-fate (Kwak et al., 2006a,b) via the IL-6/gp130 signaling pathway (Kwak et al., 2010).

In light of the role of sAPPα in CNS development, and recent studies demonstrating elevations of sAPPα in the plasma of autistic children (Sokol et al., 2006; Ray et al., 2011), we hypothesized that autism patient subsets would exhibit elevations in brain sAPPα and mice designed to over-express human sAPPα in brain tissues would reveal signs suggestive of gliosis and autism-like behavior.

### Materials and Methods

#### Human Sample Preparation

Postmortem specimens from the insular cortex brain region of 8 normally developed controls and 6 autism patients (Table 1) were obtained from through the Autism Tissue Program from the National Institute of Child Health and Human Development Brain and Tissue Bank (NICHHD, University of Maryland, Baltimore, MD). Approval for studies involving these specimens was granted by the institutional review board of the University of South Florida. Autism diagnoses were determined using the Autism Diagnostic Interview - Revised (ADI-R). Human brain samples were homogenized in 1X RIPA buffer (Cell Signaling Technology, Boston, MA) with 1% PMSF, and centrifuged at 14,000 rpm for 90 min at 4°C before storage at −80°C. Before use, samples were centrifuged at 14,000 rpm for 2 h at 4°C.

#### Enzyme-Linked Immunosorbent Assays

Human sAPPα expression in brain homogenates and cultured cells was quantified using a highly specific assay kit (IBL-America, Minneapolis, MN). Levels of sAPPα were measured for each sample in duplicate according to manufacturer’s instructions. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used for measurements of IL-6, IL-1β, IL-4, TNFα, and IFN-γ according to manufacturers’ instructions.

#### Mice and Genotyping

Tgappα mice were generated at the H. Lee Moffitt Cancer Center Animal Core Facility (Tampa, FL) by standard pronuclear injection using a 1.8 kb genomic fragment transcribing hsaAPPα into a MoPrP vector (Bailey et al., 2012). Mice were housed in a 12-h light-dark cycle, and genotyped using quantitative real-time PCR. All tissue collection and experiments were conducted in accordance with institutional guidelines and were approved by the

---

**TABLE 1: Study Populations Characteristics**

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<th>Characteristic</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>8</td>
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<tr>
<td>Age, mean (±SD)</td>
<td>7.4 (2.2)</td>
<td>7.2 (2.5)</td>
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<td>Gender, n (% female)</td>
<td>1 (16.7)</td>
<td>3 (37.5)</td>
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<tr>
<td>Nonwhite, n (%)</td>
<td>3 (50)</td>
<td>3 (37.5)</td>
<td>0.34</td>
</tr>
<tr>
<td>Postmortem interval, mean hours (±SD)</td>
<td>19.3 (7.4)</td>
<td>17.9 (8.7)</td>
<td>0.39</td>
</tr>
</tbody>
</table>
Behavioral Tests

Open Field. Spontaneous locomotor activity and anxiety in mice were assessed in a 17 inch square arena with plastic walls and floor evenly illuminated by white light. Each mouse was placed in the center of the field and allowed to explore for 10 min. Experiments were video-recorded and total distance traveled as well as time spent in center field was measured using Ethovision behavior analysis software (Noldus Information Technology, Leesburg, VA).

Social Interaction. Test mice were first habituated to the 22 × 15 inch rectangular, three-chambered polycarbonate box with divider walls containing doorways allowing access to each chamber for 10 min. Each of the two side chambers contained an empty wire cage (Galaxy Cup, Spectrum Diversified Designs, Streetsboro, OH) that was inverted and weighted down. After habituation, test mice were enclosed in the center and an unfamiliar mouse of the same strain and gender was enclosed inside one of the wire cages. The location for the “stranger” mouse was alternated between left and right sides of the box. Test mice were allowed 10 min to explore. Experiments were video-recorded and measurements of the time spent in each chamber during both phases of the task were measured and analyzed using Ethovision behavior analysis software (Noldus Information Technology).

Mouse Brain Tissue Isolation and Preparation
Mice were anesthetized using gaseous isoflurane and transcardially perfused with cold 0.01 M PBS (pH 7.4). Brains were rapidly removed and sagittally bisected. Left hemispheres were separated into hippocampus, striatum, cerebellum and cortex regions and each region was homogenized in 1X lysis buffer (Cell Signaling Technology) with 1% PMSF (Sigma-Aldrich, St. Louis, MO), centrifuged at 14,000 rpm for 15 min and stored at −80°C. Right hemispheres were fixed overnight with 4% paraformaldehyde and cryoprotected in a graded series of 10, 20, and 30% sucrose solutions, each overnight at 4°C. Right hemispheres were then embedded in Neg50 frozen section medium (Richard-Allan Scientific, Kalamazoo, MI), and sectioned sagittally on a Microm HM 550 cryostat (Thermo Scientific) with either of the following primary antibodies: mouse monoclonal anti-Aβ1-17 (6E10, 1:1,000, Covance Research Products); mouse glial fibrillary acidic protein (GFAP) (1:1,000, Cell Signaling Technology); mouse monoclonal anti-β-tubulin (1:1,000, Stem Cell Technologies, Tukwila, WA); rabbit polyclonal anti-APP C terminus (pAb 396, 1:1,000, kindly provided by S. Gandy and H. Steiner); rabbit polyclonal anti-Notch1 (1:1,000, Epitomics, Burlingame, CA); rabbit polyclonal anti-Notch 1 intercellular domain (NICD, activated Notch 1) (1:500, Abcam, Cambridge, MA); rabbit polyclonal anti-gp130 (1:1,000, Merck Millipore, Darmstadt, Germany); mouse monoclonal anti-β-actin (1:4,000, Sigma-Aldrich). After washing with ddH2O, blots were incubated for 1 h at room temperature with one of the following horseradish peroxidase-conjugated secondary antibodies: horse anti-mouse IgG-HRP linked (1:1,000, Cell Signaling Technology); goat anti-rabbit IgG-HRP linked (1:5,000, Cell Signaling Technology). Blots were developed using Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

Cell Culture
Cortical primary neurons were isolated from E14 embryos of heterozygous-bred TgAPPz dams. TgAPPz and wild-type (WT) littermate mouse embryonic brain tissues were mechanically dissociated. Both primary cortical neurons and commercially available murine neurospheres (Stem Cell Technologies, Tukwila, WA) were cultured in suspension in DMEM/F12 (Invitrogen, Camarillo, CA) containing B27 (Invitrogen), 20 ng/mL human epidermal growth factor (hEGF), and 10 ng/mL fibroblast growth factor (IGF) at 37°C in 5% CO2. Primary neuron cultures from each embryo remained separate and genotypes were identified from embryo tails using real time PCR (Bailey et al., 2012). For differentiation, neurospheres were mechanically dissociated and filtered with a 40 μm cell strainer into single-cell suspensions in DMEM/F12 containing B27, 20 ng/mL hEGF, 10 ng/mL IGF and 10% fetal bovine serum and plated in 24-well plates (Fisher Scientific) at a concentration of 1x10^4 cells per well and incubated at 37°C in 5% CO2. Human HEK293-expressed human sAPPα (hsAPPα) was generated and purified as previously described (Barger et al., 1995; Furukawa et al., 1996). N2a (murine neuroblastoma) cells (ATCC, Manassas, VA) were grown in complete MEM supplemented with 10% fetal calf serum. Cells were plated in 24-well collagen coating culture plates at a density of 1x10^5 cells per well. After overnight incubation, N2a...
cells were incubated in neurobasal media supplemented with 3 mM dibutyril cAMP in preparation for treatment. N2a cells stably overexpressing human sAPP\(\alpha\) (N2a/sAPP\(\alpha\) cells, named 6-1 cell clone) were generated via liposomal delivery using Lipofectamine 2000 (Invitrogen) of a plasmid (pcDNA3.1-sAPP\(\alpha\)-695) containing a human sAPP\(\alpha\) cDNA coding sequence based on the predicted cleavage of the 695 aa isof orm of APP (a generous gift from Dr. Steven Barger, University of Arkansas) into N2a cells followed by G418 (400 \(\mu\)g/mL) selection.

**Immunocytochemistry**

After 7 days in culture, neurospheres were gently and mechanically triturated into single-cell suspensions, plated on chamber slides at a concentration of 20,000 cells per well and incubated for 3–7 days at 37°C in 5% CO\(_2\). Cells were fixed with 4% paraformaldehyde (Fisher Scientific) in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min at room temperature, blocked in 5% horse serum for 1 h at room temperature, and incubated overnight at 4°C in 5% horse serum containing one of the following primary antibodies: monoclonal anti-\(\beta\)-tubulin (1:1,000, Stem Cell Technologies), mouse monoclonal anti-GFAP (1:500, Cell Signaling Technology). Cells were then incubated for 30 min at room temperature with fluorescein-conjugated AlexaFluor 488, AlexaFluor 594, and AlexaFluor 555 secondary antibodies (Fluorescein-conjugated IgGs, Invitrogen) at a dilution of 1:200. Images were acquired on an Olympus FV1000 Confocal microscope.

**Statistical Analysis**

Statistical differences between genotype groups were determined using one-way analysis of variance (ANOVA) for multiple comparisons. Other statistical differences were determined using the Student’s \(t\)-test. Statistical analysis for behavioral experiments was performed using GraphPad5 Analysis software. Analyses were performed on Microsoft Excel software.

**Results**

**Elevated sAPP\(\alpha\) and \(\alpha\)-CTF Levels in Brain Tissues From Autistic Children**

Previous studies report elevated levels of sAPP\(\alpha\) in the plasma of children with severe autism (Ray et al., 2011). Since sAPP\(\alpha\) is an established neurotrophic factor (Mucke et al., 1996; Turner et al., 2003), concentrations in autistic patient brains may be affected. A commercially available human sAPP\(\alpha\) ELISA kit was used to measure the sAPP\(\alpha\) concentrations in brain homogenates from autistic patients and controls. Results show that autistic patient brains contain a significantly increased level of sAPP\(\alpha\) compared to the mean level found in the brains of normally developed controls (Fig. 1A). The distributions of individual patient sAPP\(\alpha\) levels around the means show that 4 of the 6 autistic patients had sAPP\(\alpha\) levels greater than the mean sAPP\(\alpha\) levels of the controls (Fig. 1B).

In light of the nonamyloidogenic processing pathway, increased levels of sAPP\(\alpha\) in the brain should convey a corresponding increase in the levels of the APP \(\alpha\)-C-terminal fragment (\(\alpha\)-CTF). Patient brain tissue samples were analyzed by western blot using the polyclonal antibody 396 which binds the APP C-terminal fragment. The results, along with densitometric analysis, show significantly increased expression of the \(\alpha\)-CTF fragment in autistic patient brains compared to normal control brains (Fig. 1C,D). This data suggests that subsets of autistic patients have elevated brain levels of the sAPP\(\alpha\) and \(\alpha\)-CTF fragments of APP, signifying a greater inclination towards the non-amyloidogenic pathway in patient brains and a potential role for the associated fragments in the pathophysiology of autism.

**TgsAPP\(\alpha\) Mice Have Elevated hsAPP\(\alpha\) Levels in the Brain**

To identify the potential role of elevated sAPP\(\alpha\) levels on neurodevelopment, our group has studied a transgenic mouse model that overexpresses human sAPP\(\alpha\) in the brain (Bailey et al., 2012). Brain sections and homogenates from a set of 3-month-old TgsAPP\(\alpha\) littermates were studied by immunohistochemistry and Western blotting techniques using the 6E10 antibody which specifically binds human APP at the A\(\beta_{1-17}\) region. Homozygous TgsAPP\(\alpha\) (TgsAPP\(\alpha\)\(^+/+\)) mouse brains stained positive for 6E10 in both the cortex and the hippocampus, confirming the expression of the human sAPP\(\alpha\) fragment in these brain regions (Fig. 2A). Western blot analysis and ELISA measurements further corroborated the expression of the protein fragment in TgsAPP\(\alpha\) mice (Fig. 2B–E), demonstrating its absence in WT littermates and genotype-dependent levels of fragment expression in both cortex (Fig. 2B,C) and hippocampus (Fig. 2D,E) regions. These studies confirm the overexpression of hsAPP\(\alpha\) in TgsAPP\(\alpha\) mice.

**Impaired Social Behavior and Hypoactivity in TgsAPP\(\alpha\) Mice**

Social interaction deficits, increased anxiety and hypoactivity are characteristic behaviors of autism patients. To examine social functioning and motor activity in our transgenic mice, we used mouse behavioral tests for open field and social interaction (Crawley 2004).

In the open field task, mice were placed in an open box for 10 min and observed for locomotor activity and time spent in the center of the field. There was no significant difference between groups in time spent in the center (Fig. 3A); however, transgenic mice traveled significantly less distance in the apparatus than WT littermate controls (Fig. 3B), suggesting hypoactivity.

For the social interaction experiment, test mice were observed for 10 min in a 3-chambered apparatus containing an empty wire cage on one end and a wire cage enclosing a
stranger mouse on the other end. Whereas WT mice ($n=5$) spent significantly more time in the chamber containing the stranger mouse than in the chamber with the empty wire cage, there was no significant difference in the periods of time that TgsAPPα mice ($n=4$) spent in each of these chambers (Fig. 3C,D). Ratios of time spent with stranger/time spent with empty wire cage showed a decrease in time spent with stranger mice by TgsAPPα mice compared to WT littermates. This finding demonstrates a decreased preference for sociability in TgsAPPα mice compared to WT controls.

**Increased GFAP Expression in Brains of Adult TgsAPPα Mice**
An association between APP and increased GFAP expression in the brain has been firmly established in studies on patients suffering from AD and Down syndrome (DS) (Jorgensen et al., 1990; Sugaya et al., 2007). To find out whether the overexpression of sAPPα was associated with an increase in this astrocyte marker, brain sections from 3-month-old TgsAPPα mice and WT controls were subjected to immunohistochemical staining with GFAP (Fig. 4A). Other brain sections from these mice were double stained with GFAP and Nissl, which identifies cell bodies and delineates morphology in brain tissues (Fig. 4B). Image analysis and quantification of GFAP staining show that there are significantly more GFAP-positive cells in the hippocampus and entorhinal cortex of TgsAPPα mice compared to WT controls (Fig. 4C). Immunohistochemistry findings were verified by Western blot analysis on cortical and hippocampal brain homogenates and, as expected, significantly increased GFAP expression was found in TgsAPPα mice in both regions compared to WT controls (Fig. 4D). This GFAP increase is also seen in 1-month-old and 6-month-old TgsAPPα mice compared to WT controls (data not shown).

**Increased Glial Differentiation in TgsAPPα Derived Murine Neuronal Stem Cells**
Murine NSCs derived from TgsAPPα and WT E14 embryos were cultured under differentiating conditions for 3 days. Using immunofluorescence techniques, these differentiated stem cells were fixed and stained with antibodies against neuronal marker β-tubulin III and GFAP. Compared to murine NSCs from WT embryos, there was less β-tubulin III expression and greater GFAP expression in murine NSCs derived from TgsAPPα embryos (Fig. 5A). Immunofluorescence findings were verified by Western blot analysis on cell lysates prepared from mouse NSCs (Fig. 5B). As expected, significantly increased GFAP expression was found in TgsAPPα mouse-
derived NSCs (Fig. 5C). The levels of human sAPPα (hsAPPα) secreted into the media by the murine NSCs from both groups of mice were measured using a commercially available ELISA kit. The ELISA results confirmed that the hsAPPα transgene is expressed in murine NSCs from TgsAPPα embryos and that the protein fragment is secreted from these murine NSCs (Fig. 5D). Overall, these results support the association between sAPPα and increased GFAP expression in vivo.

**Increased Glial Differentiation in Wild-Type Murine NSCs Treated With Recombinant sAPPα**

Murine NSCs were treated with 2 nM concentrations of recombinant human sAPPα (rhsAPPα) under differentiating conditions composed of 2% fetal bovine serum (FBS) in complete culture medium containing growth factors. In three independent experiments using immunofluorescence staining after 5 days of culture, enhanced GFAP (green) and suppressed β-tubulin III (red) expression were observed under these conditions in murine NSCs treated with rhsAPPα compared to NSCs treated with heat-inactivated rhsAPPα (n = 3 for each culture condition) (Fig. 6A). Western blot analysis further confirmed this observation (Fig. 6B,C). These results further suggest that excess sAPPα may promote glial cell fate in isolated murine NSCs.

**Increased Expression of IL-6, NICD, and gp130 Correlated With Elevated sAPPα Levels**

Standard mechanisms of action by which sAPPα achieves its effects in the brain are still yet to be determined. With these experiments, we aimed to identify possible signaling pathways involved in sAPPα function. Previous studies implicate the IL-6/gp130 pathway and the activity of Notch1 intracellular domain (NICD, activated Notch1) in glial differentiation of NSCs (Rodriguez-Rivera et al., 2009; Kwak et al., 2010). IL-6 concentrations in cortical brain homogenates from 3-month-old WT (n = 4) and TgsAPPα (n = 4) mice were measured by ELISA. TgsAPPα mice demonstrated a significantly increased mean concentration of IL-6 per milligram of protein than WT littermates (Fig. 7A). Brain homogenates were also subjected to Western blot analysis using antibodies against human sAPPα, gp130, NICD, and β-actin. The blots exhibited greater protein expression of gp130 and NICD in the TgsAPPα mice, which verifiably express hsAPPα compared to WT littermates (Fig. 7B).

To determine whether this effect is specifically due to the presence of hsAPPα we transfected N2a neuroblastoma cells with the hsAPPα gene to create a cell line, named clone 6-1, which overexpresses the hsAPPα protein fragment. Conditioned media from triplicate cultures of each of these two cell lines were collected 18 h after plating (without treatment).
and subjected to IL-6 ELISA. The 6-1 cells secreted a significantly higher level of IL-6 than N2a cells under normal culture conditions (Fig. 7C). Lysates from each cell line were analyzed by Western blot using antibodies against hsAPP\(\alpha\), gp130, activated Notch1, and \(\beta\)-actin. Results confirm the successful transfection of the hsAPP\(\alpha\) fragment in the 6-1 line and show increased expression of gp130 and NICD in this cell line compared to the WT neuroblastoma line (Fig. 7D).

Finally, N2a cells and murine NSCs were treated with 0, 0.5, 1, and 2 nM doses of recombinant hsAPP\(\alpha\) (rhsAPP\(\alpha\)) for 24 h. IL-6 concentrations secreted into the media were measured by ELISA and the cell lysates were subjected to Western blot analysis using antibodies against gp130, activated Notch1, and \(\beta\)-actin. Although the murine NSCs secreted notably less IL-6 than the N2a cells, treatment with rhsAPP\(\alpha\) induced both types of cells to secrete increased levels of IL-6 in a dose-dependent manner (Fig. 7E). Dose-dependent increases in gp130 and activated Notch1 were also evident in cell lysates after treatment with rhsAPP\(\alpha\) (Fig. 7F).

In addition, we observed the cortical expression of gp130 in TgsAPP\(\alpha\) mice and WT controls by immunohistochemistry. TgsAPP\(\alpha\) mice exhibit increased gp130-positive staining compared to WT littermates (Fig. 8A,B). As further support, mouse brain homogenates were prepared from dissected cortical tissues and subjected to Western blot analysis. Notably, these results confirmed the increased expression of gp130 in the cortical region (Fig. 8C).

**Discussion**

This work reports the discovery of elevated levels of sAPP\(\alpha\) and \(\kappa\)-CTF in the insular cortex of autism patients and suggests increased GFAP expression-associated hypoactivity and social deficits in mice designed to over-express hsAPP in brain tissues. The work further reveals that the observed GFAP upregulation is correlated with elevations in IL-6, gp130, and Notch1. These observations support our hypothesis that subsets of patients with autism exhibit elevations in CNS sAPP\(\alpha\) and mice generated to mimic this observation display markers suggestive of gliosis and autism-like behavior.

Since the neurotrophic and neuroprotective functions of the sAPP\(\alpha\) fragment are generally accepted, previous studies revealing the presence of sAPP\(\alpha\) in autism patient plasma raise questions about the association between the APP fragment and autism pathophysiology (Sokol et al., 2006; Bailey et al., 2012). We discovered, in this study, that autism patients exhibit elevated levels of sAPP\(\alpha\) as well as notable...
increases in \(\alpha\)-CTF compared to controls in a portion of the insular cortex (Fig. 1) which is part of the gray matter in the CNS. The insular cortex was selected because it is part of the neocortex that shows neurobiological abnormalities in some autistic populations and appears to be involved in language and attention (Bailey et al., 1998; Binstock, 2001). The initial study proposing this unconventional association mentioned that the presence of the sAPP\(\alpha\) fragment could be evidence of heightened \(\alpha\)-secretase processing (Sokol et al., 2006); and our findings, particularly the dramatic increase in \(\alpha\)-CTF levels, support this inference.

APP is in fact upregulated in the brain in response to neural injury, but, as previously mentioned, sAPP\(\alpha\) plays a neuroprotective role (Mattson et al., 1993a). As Table 1 shows, several of the samples from both control and autistic cases involve causes of death that could impact brain APP levels. Some of the individual causes of death occur in both groups of cases, thereby alleviating a potential confounding variable. For example, a comparison of the levels of \(\alpha\)-CTF in control cases of drowning with the \(\alpha\)-CTF level in the autism case of drowning shows increases in \(\alpha\)-CTF expression in the control cases of drowning compared to control cases with other causes of death. However, there is an even greater increase in \(\alpha\)-CTF expression within the brain sample from the autistic case of drowning compared to the control cases.

Our data confirms that the TgsAPP\(\alpha\) mouse we generated exhibits greater expression of hsAPP\(\alpha\) in the cortex and hippocampus regions of the brain compared to WT littermate controls. In light of our observations of the autistic insular cortex,
the TgsAPPα mice mimic the human condition of elevated cortical sAPPα levels. We have previously reported that these mice also demonstrate high sAPPα levels in the plasma (Bailey et al., 2012), which also mimics the original finding in autism patient plasma (Sokol et al., 2006). It is worth noting that hsAPPα in heterozygous TgsAPPα mice is expressed at over 10 times the sAPPα levels we observed in the human condition, and homozygous mice have over 20 times greater hsAPPα expression compared to patients studied here. This exaggeration of sAPPα overexpression in the model represents a significant limitation in the translation of the results to the human condition.

Additionally, TgsAPPα mice demonstrate behavioral impairments that simulate autistic behaviors in humans. In addition to impaired social interaction, which is a cardinal autistic behavioral phenotype, patients have also demonstrated hypoaactivity (Gillberg and Billstedt, 2000; Maestro et al., 2005; Receveur et al., 2005). TgsAPPα mice exhibit reduced preference for social interaction, indicating impaired sociability (Fig. 3A). Further, in the open field task, TgsAPPα mice demonstrated hypoactive exploratory behavior (Fig. 3C, D). Altogether, the neuropathological and behavioral features of TgsAPPα mice are suggestive of components of an autism phenotype, however further studies are required to substantiate sAPPα's direct causation.

Evidence of increased GFAP and gliosis in autism patients has been demonstrated in several studies (Bailey et al., 1998; Sabaratnam, 2000; Casanova, 2006). One study shows that the level of GFAP in the CSF of autism patients was at almost three times the level normally developed patients (Ahlsen et al., 1993). Vargas et al. and Fatemi et al. report increased GFAP reactions in different regions of autistic patient postmortem brains, including the middle frontal gyrus and the superior frontal cortex (Laurence and Fatemi, 2005; Vargas et al., 2005). These observations in autism patients may be related to the longstanding theory that astrocytes participate in the brain’s immune response and indicate tissue damage (Casanova, 2006). Adult TgsAPPα mice demonstrate increased GFAP
expression in the entorhinal cortex and hippocampus regions compared to WT littermate controls (Fig. 4). These results suggest a possible role for the elevated levels of sAPPα and increased GFAP expression observed in postmortem autism brains.

There is minimal emphasis in the literature on sAPPα function in other brain cells besides neurons. Its trophic and protective effects on neurons have been well-documented (Mascal et al., 1992; Milward et al., 1992; Mattson et al., 1993b; Roch et al., 1994; Smith-Swintosky et al., 1994; Luo et al., 2001). Evidence exists supporting sAPPα activity on microglial cells, stimulating the release of interleukin-1 (Li et al., 2000), glutamate and markers of inflammation (Barger and Harmon, 1997; Barger and Basile, 2001). The cellular function of sAPPα in astrocytes was introduced with reports that treatment of human NSCs with recombinant sAPPα caused increased astrogligenesis in vitro (Kwak et al., 2006a). More recently, sAPPα has been proven to increase subgranular zone (SGZ)-derived neural progenitor cell (NPC) proliferation in culture, and treatment of these cells with sAPPα increased NPC differentiation into astroglial cells (Baratchi et al., 2011).

We observed increased GFAP expression and decreased β-tubulin III expression by primary murine NSCs derived from TgsAPPα mice (Fig. 5). We also detected dose-dependent increases in GFAP-positive cells differentiated from WT murine NSCs treated with rhsAPPα (Fig. 6), suggesting that in cell cultures sAPPα could promote glial cell fate. Despite these findings it is likely that upregulation of α-CTF itself (Fig. 1) has effects on APP and its binding partners beyond those of sAPPα. Interestingly, it is not known whether α-CTF and sAPPα can bind together and form a heterodimeric receptor as in the case of Notch1 and other similar receptors (Blumhauer et al., 1997; Furukawa et al., 1996; Van Nostrand et al., 2002; Shaked et al., 2006; Chen et al., 2006; Kedikian et al., 2010;
FIGURE 7: Increased expression of IL-6, NICD, and gp130 correlated with elevated sAPPα levels. A: Cortical brain homogenates were prepared from 3-month-old TgsAPPα mice and WT littermates (n = 4) and subjected to IL-6 ELISA (mean ± sd of pg of IL-6/mg total protein). IL-6 production was enhanced in cortical tissues from TgsAPPα mice. B: WB analysis of these tissues for sAPPα (6E10), gp130, NICD, and β-actin. C: Conditioned media collected after 18 h from N2a cells overexpressing human sAPPα (6-1 cell clone) show enhanced IL-6 production by ELISA compared to N2a cells. Data presented as mean ± SD (pg of IL-6/mg protein) from three independent experiments, with three replicates per group. D: WB analysis of these cells for sAPPα, gp130, NICD, and β-actin. WT N2a cells (E, top panel) or murine NSCs (E, bottom panel) were treated with sAPPα at doses indicated for 24 h and subjected to IL-6 ELISA revealing enhanced levels of IL-6 in sAPPα treatment groups. IL-1β, IL-4, TNFα, and IFN-γ were undetectable (data not shown). F: In parallel, the cell lysates were subjected to WB analysis using gp130 and NICD antibodies. Data presented as mean ± sd (pg of IL-6 per mg total protein from three independent experiments, three replicates per group). **P<0.01; ***P<0.001.
Hence the properties of α-CTF in subsets of individuals with autism remain elusive.

Several binding partners for sAPPα have been identified, such as apolipoprotein E (Barger and Harmon, 1997), the class A scavenger receptor (Santiago-Garcia et al., 2001), and others (Kounnas et al., 1995; Knauer et al., 1996; Das et al., 2002), providing a number of candidates that may be responsible for its cellular activities. However, none of these proteins or receptors is involved in glial differentiation. IL-6 family proteins promote the differentiation of NSCs into astrocytes and inhibit neurogenesis through activation of the JAK-STAT pathway (Bonni et al., 1997). The binding of leukemia inhibitory factor (LIF) ligand to its receptor recruits membrane gp130 to form a complex that triggers the JAK-STAT pathway (Taga and Fukuda, 2005). Additionally, Notch1 inhibits neuronal cell fate and promotes glial differentiation of NSCs via its intracellular domain (Wang and Barres, 2000; Gaiano and Fishell, 2002). In particular, Notch1 guides cells that were already destined to become glia towards an astrocytic rather than oligodendrocytic cell fate (Grandbarbe et al., 2003; Lasky and Wu, 2005). Sugaya et al. showed that sAPPα induces glial differentiation of human NSCs by activating the IL-6/gp130 pathway (Kwak et al., 2010). This group also reported that Notch1 signaling is involved in sAPPα-induced glial differentiation of NSCs (Kwak et al., 2011). Our findings confirm these sAPPα mechanisms of action in murine NSCs. We observe increased expression of IL-6 and gp130 in NSCs from TgSAPPα mice and N2A cells treated with conditioned medium from NSCs obtained from TgSAPPα mouse NSC culture. Moreover, our observation of increased NICD expression after these experiments suggests that sAPPα operates through the Notch1 pathway (Fig. 7). The sAPPα fragment may not be directly binding to the receptors that trigger either the IL-6 or Notch1 pathways; however, these findings imply that other sAPPα binding partners exist. Velasco et al. reported that stimulation of glial differentiation in rat NSCs via the Notch1 pathway is more potent than stimulation via the IL-6/gp130 activated pathway (Rodriguez-Rivera et al., 2009). Our results suggest that sAPPα can promote glial cell fate of murine neural cells.

In addition to its well-established function in glial differentiation during development, gp130 participates in the regulation of serotonergic gene expression in the mouse brain (Kulikov et al., 2010) and, along with other constituents of the IL-6 pathway, is activated in the astrocytic response to traumatic brain injury (Oliva et al., 2012). Kirsch et al. previously reported that the IL-6/gp130 pathway regulates the astrocytic response and axonal sprouting of neurons after entorhinal cortex lesion in adult rats (Xia et al., 2002). Here, we report that adult TgSAPPα mice demonstrated marked increases of gp130 in the entorhinal cortex by immunohistochemistry (Fig. 8). This suggests that the overexpression of sAPPα in the brain is associated with continually increased presence of gp130, even after development. In light of the aforementioned results (Fig. 4), this gp130 increase may correlate with the GFAP increase we observed in TgSAPPα mice;
however direct causation is lacking. Whether excess sAPP\(\alpha\) may potentially be impacting gliosis and abnormal brain development through upregulation of gp130 and consequently gp130-related pathways such as Notch1 and LIF in some individuals with autism remains to be determined.

In summary, we have shown that subsets of patients with autism exhibit elevations in sAPP\(\alpha\) and \(\alpha\)-CTF in the insular cortex compared to normally developed children. Mice generated to mimic this observation show abnormal social behavior, increased GFAP expression, and decreased \(\beta\)-tubulin III with associated elevations in IL-6, gp130, and N2a cells treated with rhsAPP and hypoactivity, increased GFAP expression, and decreased mitogen-activated protein kinase kinase and Janus kinase-signaling transducer and activator of transcription pathways in conjunction with Notch signaling. Stem Cells 26:2611–2624.


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