Human rGDF-11 counteracts age-related short-term memory impairments in middle-aged mice

Min Zhang

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Carleton University

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Min Zhang

Abstract

In humans, visuospatial memory begins to decline as early as the mid-30s, yet the mechanisms involved in this phenomenon are poorly understood. Recent research suggests that growth differentiation factor-11 (GDF-11) can have a beneficial impact on cognitive ability in old age. The mechanisms mediating this effect are unclear and there is currently no information regarding potential impact of GDF-11 on cognitive ability in the middle age years. The goal of this thesis was to explore the effects of GDF-11 treatment on the cognitive ability in middle-aged mice. Young mice and middle aged mice were treated with GDF-11 and the impact on short term memory was evaluated. The data showed significant improvement in the performance of visual memory tasks and increased neurogenesis in middle-aged mice. Taken together, these results suggest that GDF-11 is a promising candidate for combating the age-related cognitive decline associated with middle age.

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List of Abbreviations

AMH - anti-muellerian hormone

BBB - blood brain barrier

BDNF - brain derived neurotrophic factor

BMP - bone morphogenetic proteins

co-SMAD common-mediator SMAD

DCX - doublecortin

DI - discrimination index

DNA - deoxyribonucleic acid

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GASP-1/2 - GDF-associated serum protein-1/2

GDF-8 - growth differentiation factor 8

GDF-11 - growth differentiation factor 11

GDNF (glial cell line-derived neurotrophic factor

HRP - horseradish peroxidase

iSMAD - inhibitory SMAD

MAD - mothers against decapentaplegic

MAPK - mitogen-activated protein kinase

MH1/2 - MAD-homology domain 1/2

MSTN - myostatin

NGF - nerve growth factor

NOR - novel object recognition test

pAkt – phosphorylated Akt

PH3 - phosphorylated histone h3

pSMAD - phosphorylated SMAD

rGDF-11 - recombinant growth differentiation factor 11

RIPA - radioimmunoprecipitation assay buffer

R-SMAD - receptor-associated SMAD

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMURF - SMAD ubiquitination regulatory factor

TBST tris buffered saline

TGF-β – transforming growth factor beta

TOR - target of rapamycin

Introduction

Cognitive Decline with Aging

As of July 1st, 2015, the number of Canadians over 65 years of age had exceeded the number of Canadians less than 15 years old for the first time in history (StatCan, 2015). Accounting for over 16% of the population and showing a four-fold rate of increase compared to the population at large, Canada, along with most of the developed world, is playing host to an increasingly older population (StatCan, 2015). With an aging population, cognitive dementias that are prevalent in older individuals such as Alzheimer's disease have become very popular topics of research in recent years (Rowe and Kahn, 1997). A less explored but no less important topic is the cognitive decline observed in middle age, which could serve as a precipitating factor in cognitive deficits in old age. It is a generally accepted that cognitive abilities decline with age in most organisms by nature, with both animal and human studies showing significant losses in working memory, processing speed, and problem solving skills in individuals of advanced age (Park and Schwarz, 2012). These skills progressively diminish with age and can be seen as early as mid-30s (Schroeder and Salthouse, 2004, Salthouse, 2009), but most current research is focussed on either analysis of the very young or the very old; leaving much to be understood about the potential precipitating factors that could be utilized towards mitigating cognitive deficit in the middle age. This analysis of middle age cognitive ability and mechanisms to halt or reduce the early signs of cognitive deficit

during middle age is critical to promoting and prolonging cognitive ability well into old age.

As early as 1962 researchers were able to correlate significant decreases in memory tasks to aging that were independent of dementias (Kral, 1962), and in the 1980s the cognitive decline of "normal" aging was generally accepted as being dissociated from the symptoms of "pathogenic" dementias (Rowe and Kahn, 1997), though even today the distinction is difficult to classify for scientific study due to the imperfect detection of dementias (Deary et al, 2009). While natural cognitive decline was traditionally associated with advanced age starting in the 60s for humans, recent research suggests that the effects of cognitive aging can be seen as early as the late 20s and early 30s (Salthouse, 2009). Cognitive decline is not uniform across individuals in a population, with many known confounding factors such as education, environment, and even nutrition all contributing to an increased heterogeneity in cognitive abilities of humans as their age increases (Ardila, 2007). The effects of cognitive decline are not even uniform across all mental functions, for instance, verbal ability is resilient to aging and suffer little decline even at very advanced ages, while areas associated with "fluid memory", such as working memory and reasoning, decrease rapidly after the age of 30 in humans, using validated cognitive tests (Ryan et al, 2000). However, despite all these difficulties, recent comparison studies have shown a statistically significant trend in greater cognitive decline with increasing age starting from as early as age 20 and quite pronounced by the time subjects reached age 50 (Schroeder and Salthouse, 2004), and regional brain images show longitudinal declines in volume as early as 30 years of age (Fotenos et al, 2005). As studies continue to emerge presenting evidence that support the onset of cognitive decline

in early adulthood, natural cognitive aging becomes an increasingly more important area of research, as the affected individuals represent the vast majority (over 75%) of the Canadian workforce (StatCan, 2011).

Recent technological breakthroughs, specifically the development of DNA microarrays and advances in brain imaging, had given wings to the study of brain aging by allowing observation of its biological changes (Bishop et al, 2010). Though still poorly understood, the loss of cognition with aging is generally believed to be due to two main factors: disruptions in the brain system coordination between multiple regions that produce high-level cognition (Andrews-Hanna et al, 2007), and sweeping changes in gene and protein expression that is highly conserved between different animals including humans (Yankner et al, 2008). Interestingly, recent evidence suggests that young blood contains potential rejuvenation factors that could be useful in warding off the cognitive signs of aging (Villeda et al, 2014). Intriguingly, this work was facilitated by a tremendous technological advance which allowed researchers to couple circulation of a young mouse with that of an old mouse (using the so called "heterochronic parabiosis" technique). Further research into the potential factors involved in promoting improved cognitive ability in old mice lead to the identification of growth differentiation factor -11 (GDF-11), a member of the transforming growth factor beta (TGF-β) family, as a potential rejuvenation factor. The goal of this thesis research was to investigate whether GDF-11 promotes improved cognitive function in middle aged mice, and to determine the potential mechanisms involved.

Research Objectives and Major Hypotheses

Objective 1: To determine the functional impact of rGDF-11 treatment on cognitive decline in middle-aged mice, using the Y-maze and Novel Object Recognition tests.

Hypothesis 1: Intraperitoneal rGDF-11 injection will recover the deficiency in performance in working memory behavioral tests that middle-aged mice (9 month) suffer from due to cognitive decline from normal aging.

Objective 2: To determine the mechanisms used by GDF-11 in the mouse brain to promote its functional effects in middle aged and young mice.

Hypothesis 2: If GDF-11 is involved in improving cognitive ability, then increased expression of GDF-11 downstream effectors such as pSMAD2/3 and Akt will be elevated in the regions of the brain that are associated with short term working memory, such as the dentate gyrus, after GDF-11 treatment.

TGF-β

Transforming Growth Factor Beta (TGF-β) is a superfamily of growth factors with homologs found in all tested metazoans that regulate an incredibly diverse array of biological functions throughout all stages of life (Pang et al, 2011). In humans, there are currently 34 members: TGF-β1,2,3, 10 Bone Morphogenetic Proteins (BMPs), 12 Growth and Differentiation Factors (GDFs), 5 activin/inhibins, 2 leftys, nodal, and Anti-Muellerian Hormone (AMH) (Horbelt et al, 2011). Members of the TGF-β superfamily are known to play a major role in all stages of organismal development, as well as being essential to the maintenance of adult homeostasis (Horbelt et al, 2011). Furthermore, dysfunctions in the TGF-β cascades are believed to be a direct cause of a large variety of diseases.

TGF-β genes are considered oncogenic, with studies showing increased TGF-β gene expression in gliomas in the brain, as well as ovarian, gastric and esophageal cancer in test animals, and is believed to be increasing cell motility and speeding the development of malignant tumor cells (Miyazono, 2009). However, TGF-β also acts as a cell cycle arrestor at the G1 phase and has apoptotic functions that make it one of the most important tumor suppressors (Ikushima and Miyazono, 2010). TGF-β activation is cancer suppressant in the early stages of tumor development, while promoting metastasis and neo-angiogenesis at later stages, providing a confusing picture for researchers (Singh and Morris, 2010). Unfortunately, this janiform characteristic of TGF-β is not restricted to its role in cancer.

In the nervous system, the TGF-β family once again plays opposing roles. TGF-β is crucial to the development of the nervous system, and mice knock-outs of TGF-β genes suffer a range of development issues that can be as serious as the failure of mesoderm formation and subsequent death (Conidi et al, 2011). In adults, TGF-β is responsible for the upregulation of the limited amounts of neurogenesis that occurs in the sub-ventricular zone (SVZ) and olfactory bulb (OB), while at the same time being responsible for the down-regulation of neuron repair mechanisms post-injury, making it a very important, but perplexing, area of study (Mira et al, 2010; Sabo et al, 2009).

Despite being a superfamily of genes that regulate many unrelated biological processes, the cascades of TGF-β are highly conserved between members and are also relatively simple. The combination of its multiple functions, along with the ubiquitous presence of TGF-β homologs in metazoans, is one of the primary reasons for the theory that the evolution of the TGF-β cascade was the essential step necessary for complex multi-cellular life to develop (Huminiecki et al, 2009).

All members of TGF-β are formed in a pro-peptide form, with the growth factor domain non-covalently attached to a pro-domain that is removed during processing via Furin prior to secretion to create a mature peptide that is highly conserved within families of TGF-β (Pang et al, 2011). The active TGF-β protein is then secreted, and begins its cascade by binding to its corresponding cell surface receptor. There are two types of TGF-β receptors: type I and type II. TGF-β protein binds to the type II receptor, which allows the type I receptor to bind and form a three-unit complex. The type I receptor is then phosphorylated by the type II receptor at its serine/threonine kinase domain, activating it and allowing it to in turn phosphorylate the main messenger molecule-

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receptor-associated SMAD proteins (R-SMAD). Phosphorylated SMAD is able to detach from the cell membrane, and form a complex with SMAD4 in the cytoplasm. This complex is then transported to the nucleus, where it binds to DNA to control gene transcription (Horbelt et al, 2011). TGF-β proteins also produce a large variety of non-transcriptional responses, and are known to affect Erk, Akt, and MAPK, among others (Miyazono, 2000, Zhang, 2009). While all families within the TGF-β superfamily have minor differences, for example: the BMP family have higher affinity for pre-formed type I/II complexes while activins do not interact with the type I receptor at all, the cascade is highly conserved after the type I receptor becomes phosphorylated (Shi and Massague, 2003).

The TGF-β cascade is regulated both extracellularly and intracellularly. Extracellular regulation of TGF-β ligands is primarily achieved through the usage of ligand trap molecules such as Chordin and Noggin, which are in turn controlled by molecules that could cleave them to release bound TGF-β ligands, creating a multi-layered system (Balemans and van Hul, 2002). Intracellularly, the TGF-β cascade is heavily regulated, with known inhibitors of each major step, starting from the recruitment of type I receptor, which can be disrupted via the pseudoreceptor Bambi, the prevention of phosphorylation by FKBP12, the competitive inhibition of inhibitor SMADs, and the degradation of active R-SMADs by the SMAD ubiquitination regulatory factor (SMURF) (Pang et al, 2011).

As mentioned previously, the TGF-β pathway is highly conserved between different animals and between sub-families. There exist only 7 known type II receptors and 5 type I receptors, as well as 8 SMAD proteins (Massague, 2012). In addition to

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combinations of receptors and messenger proteins, other factors contribute to determine the effects of the TGF-β cascade each time it is activated to allow for such a large variety. Current doctrine has divided these factors into three types of "contextual determinants", environmental elements that shape the cellular response to the activation of the TGF-β pathway. The first contextual determinant is the concentration of each TGF-β ligand and associated elements of its specific transduction system- the effect of the TGF-β pathway as a whole is believed to be affected by the combination of each ligand successfully sending messengers into the nucleus. The second contextual determinant is the availability of SMAD cofactors: a plethora of intracellular factors exist that are necessary components to the successful transcription of genes up- and down-regulated by the TGF-β pathway, and thus the effects of a successful TGF-β cascade is in part dependent on the machinery being available to manufacture the mRNA. The third contextual determinant is the epigenetic landscape of the nucleus: throughout a cell's life cycle, different sets of genes are exposed to be affected by the R-SMAD complexes. If the binding sites for the R-SMAD complexes are not exposed, the presence of the complex in the nucleus is moot and does not affect gene expression. (Massague, 2012) Current research indicates a very complex and multi-faceted control system for the comparatively simple TGF-β cascade. Combined with its seemingly ubiquitous presence in the biological functioning of all metazoans, makes the members TGF-β superfamily some of the most important molecules to understand from both a theoretical and clinically perspective. Significant research has been dedicated toward evaluating the impact of TGF-beta family members on nervous system function (Flanders et al, 1991, Aigner and Bogdahn, 2008). However, given the mechanistic complexities associated with function of individual members, there

is the need to empirically determine mechanisms mediating the effects of different TGF-beta members on nervous system function. In this thesis, I present evidence which supports a role for one family member, GDF-11, in improving cognitive function in the brain of middle aged mice.

GDF-11

  The Growth Differentiation Factors (GDFs) are a subfamily of TGF-β, so named due to their regulatory effect on organismal development in both vertebrates and invertebrates (Herpin et al, 2004). GDF-11, the 11th protein to be identified as a GDF, utilizes a pathway similar to TGF-β1/2/3 proteins (See Figure 2). GDF-11 had two known functions: as a protein necessary for development by controlling anterior-posterior patterning, kidney development, and closure of the palate (Andersson et al, 2006), and as a regulatory factor of neurogenesis that can be both positive and negative, depending on the extracellular environment (Gamer et al, 1999; Wu et al, 2003). GDF-11 is very closely related in structure to GDF-8, commonly known as Myostatin (MSTN), a TGF-β protein primarily responsible for the negative inhibition of skeletal muscle growth and metabolism (Huang et al, 2011). Though GDF-11's mature domain has a 90% homology to GDF-8's domain, the two are known to have different expression regions and functions in different systems (Nakashima et al, 1999; Gamer et al, 1999). Recent studies suggest that the different profiles of GDF-11 and GDF-8, despite their mature domain structural similarity, is due to an extra step in their activation that is not shared by the rest of the GDF family (Ge et al, 2005). Unlike other GDF members, GDF-11 and GDF-8 proteins form a non-covalent complex with their pro-domain after their precursors are cleaved (Figure 1), and remain latent until further processed by a tolloid-like metalloproteinase (Ge et al, 2005, Walker et al, 2016). Unlike the mature domains, the prodomains of the two proteins share only 52% homology and create different structures with their mature proteins, and is theorized to be why GDF-8 and GDF-11 have such different functions despite their similar amino acid composition (Walker et al, 2016). Despite being labeled

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as a BMP due to its localization and effect on skeletal development, GDF-11 shares traits with both the TGF-β1-3 proto-proteins (Ge et al, 2005) and activin subfamily (Gamer et al, 1999), and is known to be expressed in select regions of the brain, unlike most other BMPs (Nakashima et al, 1999). GDF-11 was thrust into the spotlight in 2013 and quickly became a protein of intense interest for society at large, when a series of studies conducted at Harvard University published results that suggested that GDF-11 may have anti-aging effects in both cardiac skeletal muscle and age-related cognitive decline (Loffredo et al, 2013; Villeda et al, 2014).

Heterochronic Parabiosis

Parabiosis, derived from the Greek words 'para' (next to) and 'bios' (life), describes the state of two distinct organisms living with a surgically joint circulatory system, so that blood-flow is shared between them (Conboy et al, 2013). It was pioneered by Alex Carrel at the beginning of the 20th century, who won a Nobel Prize for his success in surgically connecting the blood vessels of cats and dogs through the removal of several organs into one visceral organism (Carrel, 1913, Conboy et al, 2013). Parabiosis techniques were improved significantly throughout the 20th century, and currently, animals can be joined via skin and joint grafts that do not trigger immunological reaction due to the sharing of blood-circulated antigens (Kamran et al, 2013). Heterochronic parabiosis, specifically, refers to a type of parabiosis experiment where two animals of different ages are joined together, to test for aspects of aging and age-related diseases (Conboy et al, 2013).
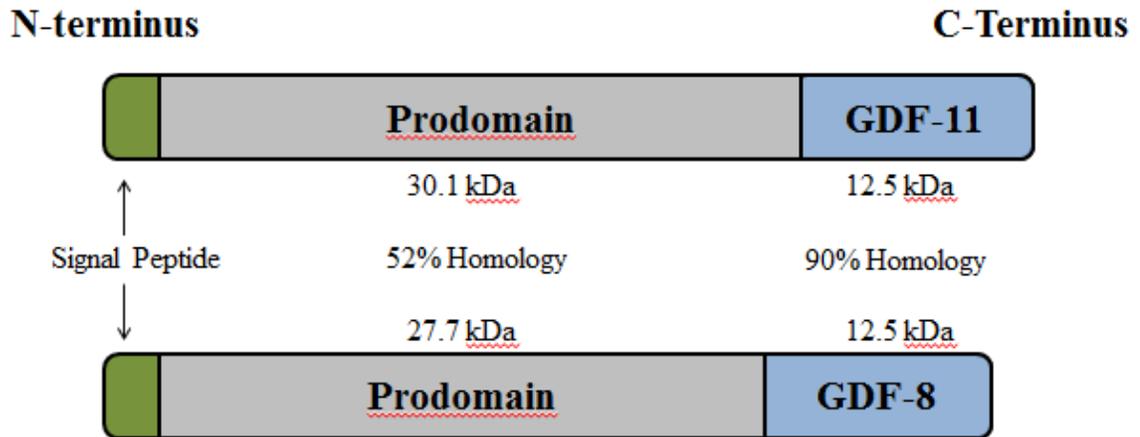
Figure 1: GDF-8/11 Precursor Protein Structure. Mature GDF-8/11 proteins are excised from their precursors via signal peptidase and furin-like proteases (Walker et al, 2016). After cleavage, the prodomains of GDF-8/11 non-covalently bind to their mature proteins, forming structurally different latent complexes that require further factors to activate. The structural difference between complexes due to differences in the prodomains is thought to be the reason GDF-8 and GDF-11 have different expressions and effects despite a high homology.

GDF-11 in Aging Research

In 2013, inspired by the potential rejuvenating effects of parabiosis with young

mice on the cardiovascular system of aged mice, Loffredo et al attempted to isolate the

blood-borne factor that was responsible for this recovery. They discovered that a

prolonged treatment of recombinant GDF-11 at the rate of one 0.1mg/kg intraperitoneal

injection per day, they could detect increased GDF-11 protein levels in the blood of

treated mice, and that after one month of treatment, the mice that were in advanced age

(23 months old) showed significant reversal of age-related heart hypertrophy- a

significant decline in the thickening of heart walls that negatively affects the

cardiovascular system of aged mice and humans that brought the heart physiology back

to youthful levels (Loffredo et al, 2013). Following this success, work from the lab of Dr.

Amy Wagers at Harvard University were interested in the effects of parabiosis on

cognition and discovered that the injection of young mouse blood into 18 month old aged

mice produced significant improvements in cognitive memory tasks such as fear response

and radial arm water maze (Villeda et al, 2014). To the excitement of the scientific

community, it was later suggested that GDF-11 treatment was sufficient to improve

neurogenesis in the brain in the olfactory bulb and sub-ventricular zone, as well as

improve an aged mouse's performance in an olfaction assay behavior test. These data

suggest that GDF-11 was not only able to facilitate neurogenesis in the central nervous

system, but that the newborn neurons were able to properly integrate into the established

neuronal network (Katsimpardi et al, 2014). This series of discoveries led to much

mainstream attention, and GDF-11 was the newest "elixir of youth" for the popular media

(Andersen and Lim, 2014). However, recent research has made these claims quite

controversial, and the direct effect and mechanisms of GDF-11 in the brain are still unknown.

Traditionally, GDF-11 was known as an inhibitory protein, similar to its homolog GDF-8 (Wu et al, 2003). With the findings of the Wagers and Rubin labs contradicting this previous data, the lab of Dr. Glass performed a series of experiments to attempt to study the effects of GDF-11 in aging skeletal muscle (Egerman et al, 2015). They showed that neither GDF-11 protein nor mRNA decreased with age in skeletal muscles, but rather increased (Egerman et al, 2015). Most damning, they demonstrated that the antibody for GDF-11 used by the above-mentioned labs (ab12741) was not specific to GDF-11, but was also able to bind GDF-8 (Egerman et al, 2015). The Glass et al paper threw the findings of Wagers and Rubin labs into doubt, and in the past year, several papers have emerged from other labs that attempt to replicate the original results, with mixed results (Poggioli et al, 2015; Smith et al, 2015; Rodgers and Eldridge, 2015). Several laboratories are now actively investigating the mechanisms used by GDF-11 to promote its effects in both muscle and nervous system. As discussed previously, one of the major downstream targets of GDF-11 is a group of molecules called SMADs.
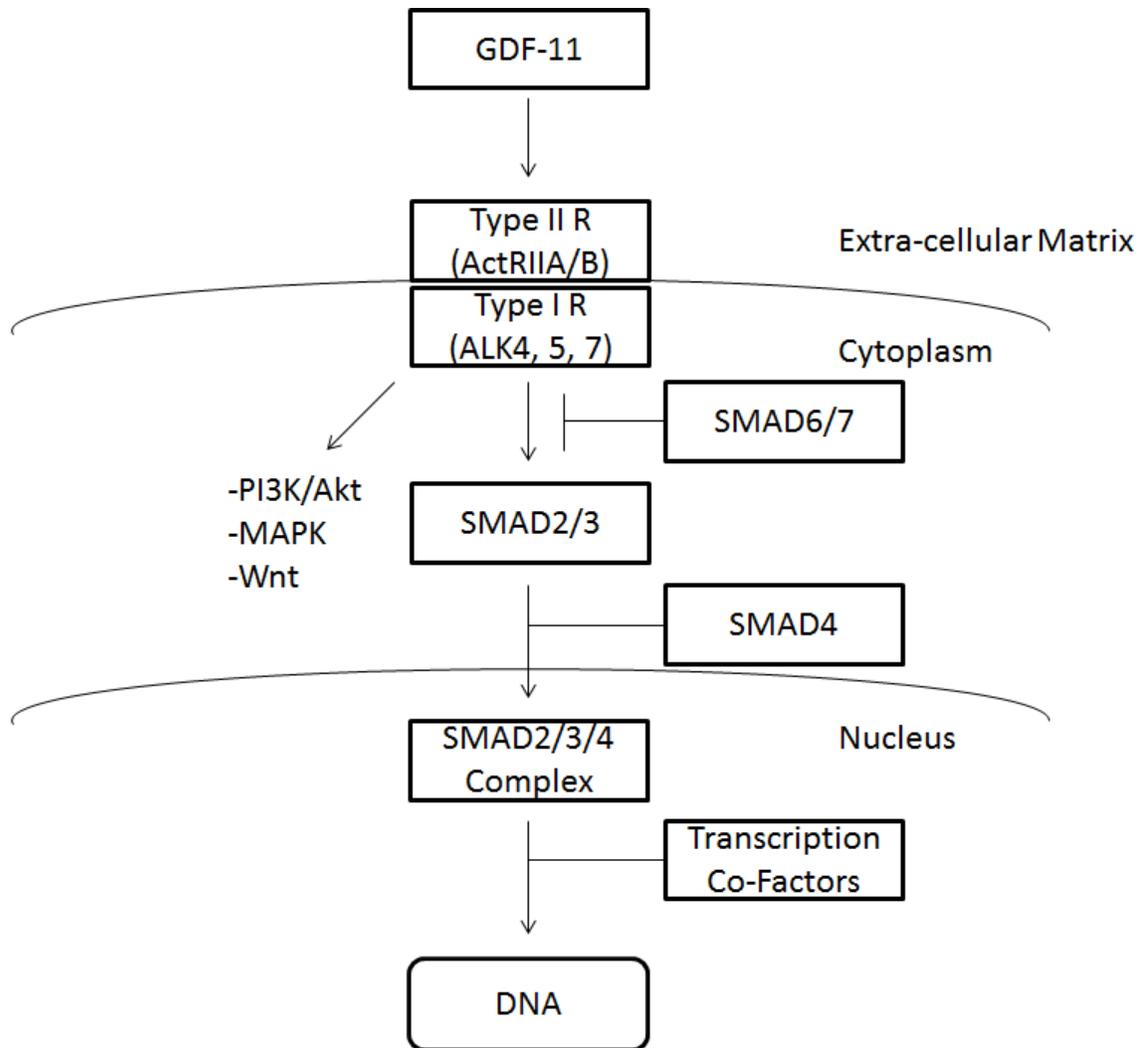
Figure 2: GDF-11 pathway. GDF-11 protein binds to the extra-cellular region of Type II receptor in the cell membrane, causing a change in configuration of the Type II receptor that allows the binding of the Type I receptor, forming a complex. The Type I receptor is then phosphorylated, which exposes its SMAD binding domain, allowing SMAD2/3 to bind and become phosphorylated. SMAD6/7 competitively binds to Type I receptors as inhibitors. Phosphorylated SMAD2/3 then complex with SMAD4, allowing it to enter the nucleus. In the nucleus, the SMAD2/3/4 complex recruits transcription co-factors to affect DNA transcription.

SMADs

The SMADs are a group of proteins that act as the messenger molecules for the TGF-β pathway. The name "SMAD" is a combination of the acronyms for the Drosophila and C. elegans protein homologs that were discovered first: mothers against decapentaplegic (MAD), and small body size (SMA), respectively. The SMAD family is composed of 8 members, and are further sub-divided into 3 types.

The first and most numerous type is the receptor-associated SMADs (R-SMADs), which encompasses SMADs 1, 2, 3, 5 and 8. SMADs 1, 5, and 8 are associated with the BMP sub-family, while SMADs 2 and 3 are used as the messenger for the other sub-families. The second type is the inhibitory SMADs (I-SMADs), which include SMADs 6 and 7. I-SMADs participate in the TGF-β cascade as negative regulators, by competitively binding to phosphorylated type I receptors to prevent the phosphorylation of R-SMADs. The last type of SMAD is the common-mediator SMAD (co-SMAD), consisting of only SMAD4. Co-SMAD binds with R-SMADs to create a complex that can enter the nucleus and participate in signalling. (Shi and Massague, 2003)

SMAD proteins are generally around 500 amino acids in length, and have two domains: MAD-homology 1 (MH1) and MAD-homology 2 (MH2). MH1 displays sequence-specific DNA binding capability and is believed to be the primary method of affecting the DNA. MH2 is a highly conserved domain among all SMADs that is responsible for receptor interaction, by both increasing binding affinity via the L3 loop (Wu et al, 2003), as well as identify specific receptors primarily via the combined shape of the L3 and L45 loops (Huse et al, 2001). R-SMAD is activated via the phosphorylation

of 2 C-terminal serine residues in the MH2 domain. The MH1 domain is active in activated R-SMADs and co-SMADs, and negatively regulates the function of the MH2 domain to ensure that active R-SMADs and co-SMADs will not bind to the type I receptor and derail the TGF-β pathway. Inversely, the MH1 domain has no DNA binding capability in I-SMADs and do not restrict MH2 activity, allowing them to continue to competitively inhibit R-SMADs. (Shi and Massague, 2003) After the R/Co-SMAD complex enters the nucleus, gene targeting and transcriptional control is carried out via cooperation between the SMAD complex and SMAD-responsive enhancers present in the nucleus- molecules with SMAD binding elements (SBEs). MH1 is designed with a low DNA binding affinity, thus multiple SMAD complexes must work together to bind to the targeted gene, while multiple MH2 domains are necessary to attract basal transcriptional machinery, which is yet another regulatory step that ensures transcriptional changes occur only when there is sufficient stimulus (Shi and Massague, 2003). R-SMAD signalling is ended via de-phosphorylation and ubiquitination via SMURF (Zhu et al, 1999). Deregulation of the TGF-β/SMAD pathway can lead to neuronal degeneration, and has been implicated in age-related cognitive deficits in animal models (George et al, 2006). GDF-11 is a TGF-β protein that has been shown to activate specific SMAD cascade in the brain, and recent studies suggest it may be able to improve the physiology of the aging brain via this downstream effector (Katsimpardi et al, 2014). However, GDF-11 activation may also influence other cellular mechanisms, including the Akt pathway (Zhang, 2008).

Akt/Target of Rapamycin (TOR)

One important pathway of note for brain aging is the Akt-TOR pathway. The TOR protein is an orthologue of yeast Tor1 and Tor2 proteins, and is highly conserved among mammals (Brown et al, 1994). TOR stands for "target of Rapamycin", and serves a large number of roles in cellular survival and proliferation, including the regulation of protein translation and mitotic cell cycle arrest (Bjornsti and Houghton, 2004). TOR signalling is shown to affect both normal and pathological aging- decreased TOR signalling had been shown to increase lifespan in mouse models (Harrison et al, 2009), and treatment with the TOR inhibitor rapamycin had been shown to inhibit Huntington's disease progression in multiple animal models (Ravikumar et al, 2004). Regulation of TOR expression is complex and multi-faceted, but a well-known and important pathway of TOR activation is the phosphorylation of the protein Akt to form pAkt, which is able to inactivate negative regulators of TOR such as rapamycin (Bjornsti and Houghton, 2004). Recent studies have shown that a superfamily of growth factors known as TGF-β were, in addition to their effects on gene transcription, an important regulator of Akt expression in the cytoplasm (Goraksha-Hicks and Rathmell, 2010). The relationship between GDF-11 and Akt is complex and not well understood, as it can promote and inhibit the pathway both directly and indirectly, depending on the organismal age and local cellular environment (Wu et al, 2007, Aigner and Bogdahn, 2008, Zhang, 2008, Brun and Rudnicki, 2015).

Assessing Cognitive Decline in Middle Aged Mice

While human studies are preferable due to the direct interpretation and application of the results to society, animal studies have some noticeable advantages; chief among them, the ability to administer novel substances that affect the central nervous system, and the direct access to brain samples that would not be possible for human experiments. Mouse models have always been a strong candidate for cognitive behavioral testing, with many previous studies lending credence to their usefulness in the investigation of behavioral alterations and brain functions (Ohl et al, 2003).

Specifically for cognitive testing of memory tasks, the C57Bl strain is an easily accessible mouse model that has also show cognitive decline in normal aging that mimics progression in humans. Past studies looking to determine the cognitive ability of C57Bl mouse strains have noted significant decline in object memory between young (3-6 month old) mice and middle aged (9-12 month old mice), with significant neurotransmitter differences between age groups (Gower and Lamberty, 1993). A 9-12 month old mouse is roughly equivalent to a 30-40 year old human (Dutta and Sengupta, 2015), which is very similar to the age groups where recent studies have detected noticeable declines in fluid memory performance tasks (Salthouse, 2009), suggesting the C57Bl strain to be a good model for experiments involving behavioral alterations in cognitive tasks between age groups. As an easily accessible animal model with significant differences in cognitive behavioral tests between young and middle-aged specimens due to normal aging, the C57Bl mouse strain makes for a good candidate to study the cognitive effects of GDF-11 between subjects of young (state how old here) and middle aged (state how old) mice.

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Materials and Methods

Animals

All procedures were conducted within guidelines as set by the Canadian Council on Animal Care (CCAC) and approved by the Carleton University Animal Care Committee. 3 week old male wild-type C57Bl/6 mice were obtained from Charles River Laboratories and acclimated for 2 weeks before experiments and tested at 1.5 month of age. 8 month old male wild-type C57Bl/6 retired breeder mice were obtained from Charles River Laboratories and acclimated for 1 month before experiments and test at 9 month of age. All mice were housed individually with 8AM-8PM light-dark cycle, with bedding and basic housing, food and water provided *ad libitum*.

Experimental Design

After the acclimation period, all mice were tested via Y-maze and Novel Object Recognition tests to establish baseline performance for each age group (see Figure 3 for timeline). Experiments were carried out with one week in between to minimize the effect of experiment repetition as a confounding factor. After one week, half of the mice from each age group were randomly chosen and given an intraperitoneal (i.p.) injection of 0.1mg/kg of recombinant GDF-11 protein (Peprotech, 120-11). All mice which did not receive the GDF-11 treatment were given an i.p. injection of saline of equivalent volume. After 24 hours, all mice were tested using Y-maze and Novel Object Recognition test using a new set of objects. Mice were sacrificed at the end of behavioral testing using

pentobarbital as anesthetic. Animals were intracardially perfused with 1x phosphate

buffered saline (PBS). The brains will then be extracted for further analysis by Western

Blot and Immunostaining. See Figure 3 for experimental timeline. Experiment groups

include two independent cohorts of 20 mice, with the distribution as follows for each

cohort (n=20 per cohort, 2 cohorts): 1.5 month + saline (n=5), 1.5 month + GDF-11 (n=5),

9 month + saline (n=5), and 9 month + GDF-11 (n=5). In all 10 mice per treatment group

were analyzed.

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|-------1 week-------|----24 hours----|----6 hours----|
↓                    ↓                ↓               ↓
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Baseline Test
-Y Maze
-Novel Object
Recognition

Injection
-0.1mg/kg
-Intraperitoneal
-Growth
Differential
Factor 11 OR
Saline

Behavioral
Test
-Y Maze
-Novel Object
Recognition

Sacrifice
-Half brain
preserved
using 4% PFA
-Half brain
frozen at -
80°C
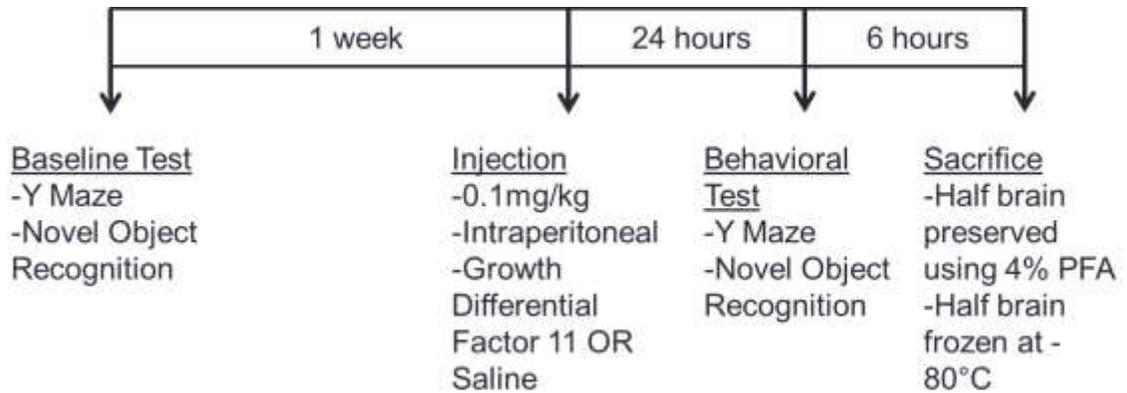
Figure 3: Experimental timeline of behavioral testing. Baseline tests are carried out after minimum 2 weeks after arrival to facility for acclimation, then after one week of no testing to remove previous testing bias, mice are injected intraperitoneally with either rGDF-11 at 0.1mg/kg or saline solution. After 24 hours to allow dispersal mice undergo the behavior tests under same parameters, then sacrificed and brains preserved for protein analysis.

GDF-11 Injection

GDF-11 treated mice received intraperitoneal (i.p.) injections of rGDF-11 (Peprotech 120-11) dose of 0.1mg/kg per mouse. Saline treated mice received i.p. injections of saline at an equivalent volume to rGDF-11 injection.

Behavioral Testing

Y-Maze

The Y-maze is a short-term spatial memory assessment task (Kennard and Woodruff-Pak, 2011). The maze is a three-arm construct made of grey plastic. Mice were placed facing the end of arm 1, and allowed to move freely within the maze for a period of 8 minutes. The sequence of arm entries for each mouse was recorded. An arm entry was defined as the hind legs of the mouse entering the arm. An alternation was defined as a series of entries into each of the three arms with no repetition. The percent alternation were the calculated of value of the ratio between observed alternations and the total possible number of alternations (defined as the number of entries minus 2), multiplied by 100.

Novel Object Recognition

Novel Object Recognition (NOR) is a visual object memory assessment task (Bevins and Besheer, 2006). The test was conducted using a featureless cage, with the objects placed equidistant from the center and the mouse placed the same distance from both objects and facing the wall. The objects were chosen to be similar in size, but different in shape and colour. During the experiment, the mouse was first be exposed to two identical objects (Objects A) for 10 minutes and allowed to explore freely. After the training session, the

mouse was returned to its habitation for an hour. After an hour elapsed, one of the Objects A was exchanged for a novel object (Object B), and the mouse was returned to the same testing cage and allowed to explore freely for 5 minutes. The amount of time a mouse spent exploring each object was recorded. A mouse was considered to be exploring an object when it was contacting it with its mouth, nose, or paw, with the exception of contacts that are judged to be accidental, or standing on top of the object. A mouse was also considered to be exploring an object when its nose was directed at the object when its nose is within 3cm. Collected exploration data was verified by a second observer to minimize the impact of observer bias. Relative exploration time was assessed using the Discrimination Index (DI), calculated by: (time novel – time familiar) / (time novel + time familiar). A positive DI indicated more time spent exploring the novel object, while a negative DI indicated more time spent exploring the familiar object. A score of 0 indicated that the mouse spent the same amount of time exploring both objects.

Brain Tissue Analyses

Brain Tissue

The right hemisphere of each brain was placed in 4% paraformaldehyde for 48 hours post-extraction, 24 hours in 10% sucrose solution, and was preserved in 30% sucrose solution until sectioned into 30μm sections using a cryostat. The left hemisphere of each brain was frozen and kept at -80°C post-extraction, and was used for Western Blotting.

Immunofluorescence

Tissue samples were collected on slides that were washed in 1x PBS.

Immunofluorescence was performed on samples using the following primary antibodies: GDF-11/Myostatin (1:250, AbCam), Myostatin (1:250 Millipore), BDNF (1:250, AbCam), GFAP (1:100, Santa Cruz) phospho-SMAD2/3 (1:300, Santa Cruz), Doublecortin (DCX) (1:200, Santa Cruz), phosphorylated Histone H3 (PH3) (1:200, Millipore) which were diluted in 0.5% Triton X-100 solution, overnight at 4°C. Sections were then washed in 1x PBS before a 2 hour incubation with anti-mouse Alexa Fluor 555 (1:200, Cell Signaling Technology) or donkey anti-goat 488 (1:200, Santa Cruz) secondary antibodies at room temperature in darkness. After washing with 1x PBS the samples were incubated with DAPI (1:10,000 Cell Signaling Technology) for 5 minutes. The samples were washed one final time with 1x PBS and cover-slipped using Fluoromount-G (Sigma).

Western Blotting

Protein extracts of the left brain hemisphere were prepared using a handheld homogenizer in RIPA buffer. SDS-PAGE was conducted on extracts to separate proteins, loading 50uL of protein from whole brain lysate to investigate protein levels within the brain for each sample. Primary antibodies were: GDF-11/GDF-8 (1:500, AbCam), GDF-8 (1:500, Millipore), phosphorlyated-Akt (pAkt) (1:1000, Santa Cruz), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:50000, Santa Cruz), diluted in 5% milk in Tris-buffered saline (TBST). Primary antibody incubation was done overnight at 4°C on an electronic shaker. Secondary antibodies to be used were horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (1:20000, Santa Cruz) and goat anti-mouse (1:20000, Santa Cruz). Detection was achieved using the SRX-101A developer (Konica), and bands were normalized using GAPDH. Bands were quantified by the following formula:

(Protein band strength/GAPDH band strength)*(Average band strength of group/Average band strength of control group)/(Average band strength of group)

Statistical Analysis

Statistical analysis of Western Blots was carried out via 2-factor analysis of variance (ANOVA) to determine interaction between the Age (1.5-months or 9-months) and Treatment (GDF-11 or Saline), and independent t-test was used when comparing differences between groups of same Treatment but different Age. Bonferroni post-hoc testing was carried out to determine the differences among the four treatment groups: 1.5months/Saline, 1.5months/GDF-11, 9months/Saline, and 9months/GDF-11. All significance were found to at least $p < 0.05$.

Results

Significant difference in performance observed in short-term visual and spatial memory tests between mice of young and middle age

Two types of behavioral tests were conducted for this experiment; novel object recognition (NOR) and y-maze, to examine short-term visual memory and spatial memory in young (1.5-months old) and middle aged (9-months old) mice.

Novel object recognition is a validated behavioural test that is frequently used in the literature to evaluate short term memory in mice (Besheer and Bevins, 2006). Mice prefer to explore novel objects in a familiar environment, and their ability to discriminate between a new, novel object from a previously presented familiar object is used in this test to quantify their memory ability. The ratio of time spent interacting with a novel object over a familiar object is represented as the discrimination index (DI), with a positive DI suggesting that the mouse spent more time with the novel object, a negative DI suggesting that the mouse spent more time with the familiar object, and a DI of 0 suggesting no behavioral preference between objects. Mice were subjected to the NOR test between 24-28 hours after treatment, when GDF-11 protein levels still remained high in the bloodstream (Loffredo et al, 2013), to determine the effects of GDF-11 injection on short-term visual memory. Independent t-test of baseline DI for the two age groups showed a significant difference, with 1.5 month-old mice spending significantly more time with the novel object compared to the familiar object than 9 month-old mice ($t_{(38)} = 6.691$, $p < 0.001$, n = 40) (Figure 4A). The observed difference between age groups

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suggests that the NOR test can reflect decline in visual short-term memory between mice of 1.5 months and 9 months of age.

Y-maze is a test designed to assess the short-term spatial memory of mice (Kennard and Woodruff-Pak, 2011). Mice are naturally inclined to explore down paths they have not taken, and this test quantifies their ability to successfully choose a corridor they have not yet explored as a percentage to judge their spatial memory. Each time a mouse successfully enters each of the 3 arms in turn without repetition it is counted as one 'alternation'. The amount of alternations a mouse makes over the total possible number of alternations it can make is then converted to percentage to produce "percent alternations" to quantify the animal's short-term spatial memory. A percent alternation score of 100 is a perfect performance, suggesting the mouse was able to remember its previous entries at all times, with lower scores suggesting progressively weaker memory. Independent t-test of baseline percent alternations for the two age groups show no significant differences between the two age groups $t_{(38)}$=1.537, $p > 0.05$, n = 40 (Figure 4B). While the data was not statistically significant, a noticeable trend toward a decline in percent alternations with age was observed, and baseline results suggest that the Y-maze used did not represent sufficient difficulty as a short-term spatial memory task to show significant differences between 1.5 months and 9 months old mice.
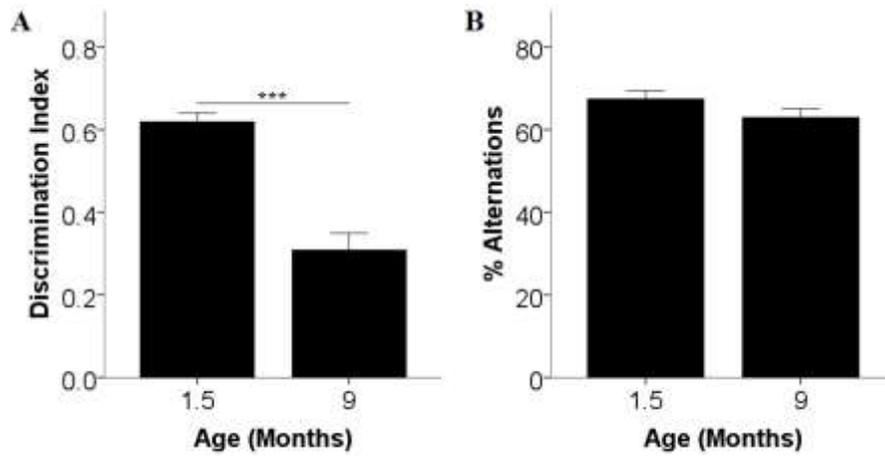
Figure 4: Baseline Behavior Performance Data. A) NOR discrimination index. 1.5 month old mice performed significantly better by spending more time with the novel object compared to the familiar object. B) Percent alternation on Y-maze. No significant difference we observed between the two age groups. ***$p < 0.001$, independent t-test.

rGDF-11 injection significantly improves short-term visual memory in treated middle-aged mice, but not short-term spatial memory

In order to evaluate the impact of rGDF-11 administration on mice short-term memory, all mice underwent a NOR and Y-maze testing 24 hours after treatment. Supported by the Grubb's test, 2 of the 40 animals tested were excluded from calculation for NOR test results post-injection, due to not moving from their original location during the entire test and therefore having no interaction with the objects, resulting in a final n = 38.

Graphical representation of the NOR test results are shown in Figure 5A. Two-way ANOVA test showed an interaction between age and GDF-11 treatment ($F_{(1,34)}$ = 9.0, p < 0.01, n = 38). Further analysis via Bonferonni pair-wise comparison revealed that GDF-11 treated middle-age mice (n = 8) spent a significantly higher amount of time interacting with the novel object than their saline-treated counterparts (p < 0.001, n = 10). Interestingly, GDF-11 treated young mice (n = 10) did not show a significantly higher DI than saline treated young mice (p > 0.05, n = 10). This suggests that visual memory, which naturally declines with age, is being restored by the GDF-11 treatment.

Figure 5B shows the percent alternation score of the four treatment groups (n = 40). Two-way ANOVA showed no interaction between age and treatment ($F_{(1,36)}$ = 1.4, p > 0.05, n = 40), and no main effects were observed for mouse age ($F_{(1,36)}$ = 0.1, p > 0.05, n = 40) or treatment ($F_{(1,36)}$ = 1.7, p > 0.05, n = 40). While it is possible that GDF-11 does not affect the spatial memory of mice at this dosage, it is likely that spatial memory did not deteriorate to a significant enough degree in middle-aged mice for the y-maze to detect differences.
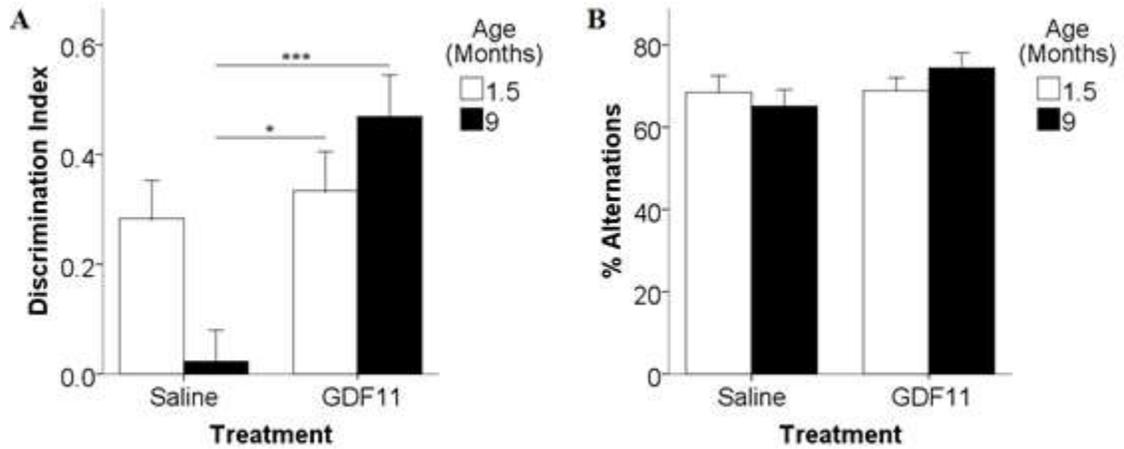
Figure 5: Post-GDF11 injection behavioral task performance. A) NOR discrimination

index graph. Significant increase detected between 9-months old saline-injected mice and

GDF-11 treated mice. B) Percent alternation made in the Y-maze. No significant

differences found.

*$p < 0.05$ and ***$p < 0.001$, Bonferroni post-hoc pairwise comparison.

GDF-8+11 protein levels are significantly higher in the brains of treated young mice

It is important to determine whetherGDF-11 expression is increased in the brain by the intraperitoneal GDF-11 treatment. In order to evaluate the expression of GDF-11, an antibody previously established to stain for GDF-11 that was used by several published papers (Loffredo et al, 2013, Katsimpardi et al, 2014) was used. It must be noted however that this antibody, not only detects GDF-11, it also detects GDF-8, a close family member. Western blot representative image and staining results for GDF-8/11 in whole brain lysate are shown in Figure 6. Two-way ANOVA detected an interaction between age and treatment ($F_{(1,8)} = 25.4$, $p < 0.001$, n = 5 - 6). GDF-11 treated young mice showed a significant increase in GDF-8+11 protein levels in the brain compared to all other treatment groups ($p < 0.001$, n = 5), while GDF-11 treated middle-age mice showed no significant change in protein level compared to the saline treated mice ($p > 0.05$, n = 5).
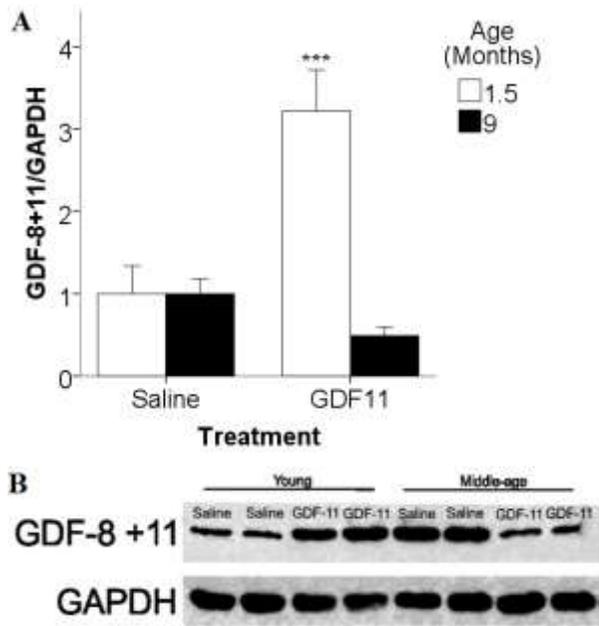
Figure 6: GDF-8+11 expression in whole brain lysate. A) GDF-8+11 western blot data after normalization via GAPDH. 1.5-months old mice treated with rGDF-11 show significant higher expression levels than other groups. B) Representative western blot of GDF-8+11. ***$p < 0.001$, Bonferroni post-hoc pairwise comparison.

GDF-8 expression in the dentate gyrus and cortex is unchanged between groups

The antibody used was recently shown to also stain for GDF-8 (Egerman et al, 2015), which we have accounted for by also determining the expression levels of GDF-8 at key brain regions known to be associated with visual memory. The results for GDF-8 immunofluorescence at the dentate gyrus and cortex are shown in Figure 7. No significant difference in GDF-8 cell numbers was detected in either the dentate gyrus ($t_{(7)}$ = 1.2, *p* >0.05, n = 4 – 5) or cortex ($t_{(7)}$ = 0.6, *p* >0.05, n = 4 – 5). This gives confidence that the protein level changes detected in the Western blot were due to GDF-11, and not the result of non-specificity due to GDF-8.
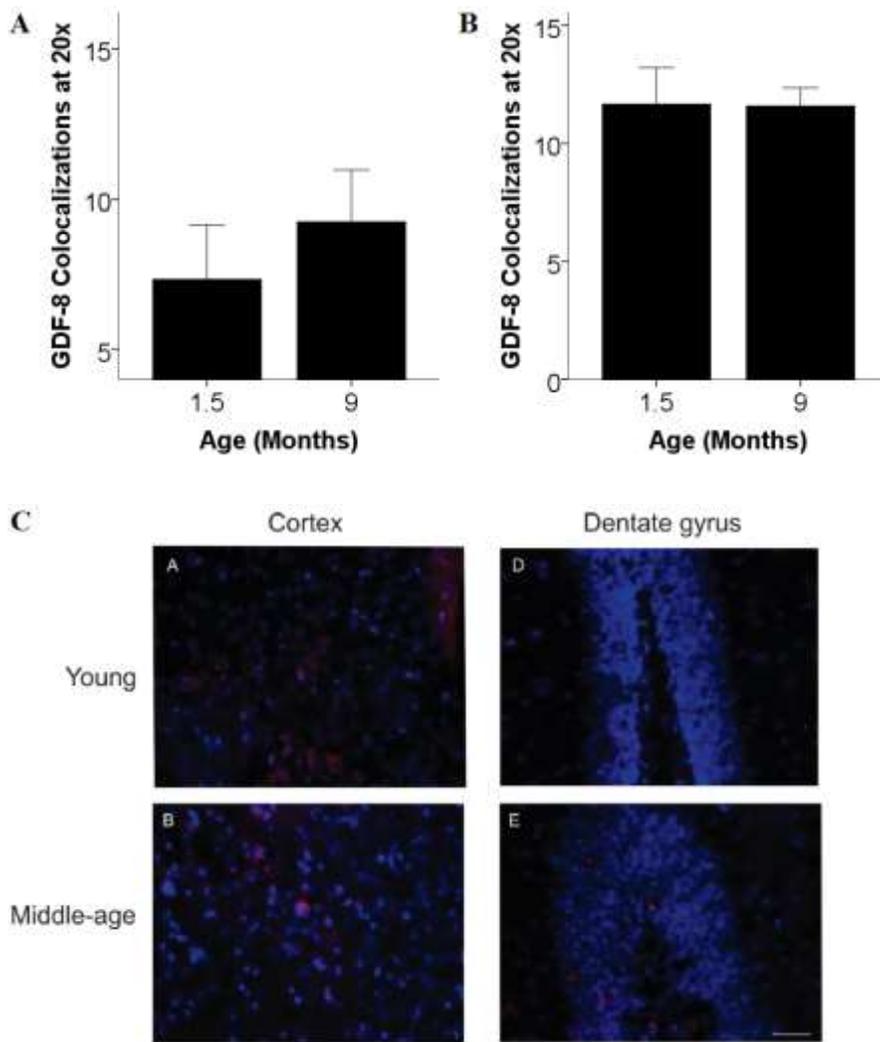
Figure 7: GDF-8 expression in the cortex and dentate gyrus. A) Quantification of

GDF-8 expression in the cortex. B) Quantification of GDF-8 in the dentate gyrus. C)

Representative 20x image of GDF-8 (red) over DAPI (blue).

Increased pSMAD2/3 expression in brain regions for treated young mice

Phosphorylated SMAD2/3 (pSMAD2/3) is the most well-known downstream effector of GDF-11, and was one of the downstream targets explored in effort to determine the mechanisms behind the improved short term memory. pSMAD2/3 expression was quantified in the dentate gyrus (DG) and cortex, which are regions well known to be associated with short-term memory processing (Squire, 1992). Results of the immunofluorescence experiments are shown in Figure 8. Two-way ANOVA determined a significant interaction in both the DG $(F_{(1,13)} = 11.0, p < 0.01, n = 6)$ and cortex $(F_{(1,13)} = 18.2, p < 0.001, n = 6)$. Bonferonni pairwise comparison showed significantly higher pSMAD2/3 expression levels in the DG of young mice treated with GDF-11 than both saline treated young mice $(p < 0.01, n = 6)$ and GDF-11 treated middle-age mice $(p < 0.05, n = 5 - 6)$. In the cortex, GDF-11 treated young mice showed significantly higher pSMAD2/3 expression levels than all other treatment groups $(p < 0.001, n = 6)$. This data suggests that the increased GDF-11 protein levels observed previously is resulting in increased activation of the GDF-11 pathway. There was however an improvement in visual memory observed in the GDF-11 injected middle-age mice, which did not show noticeable increase in pSMAD2/3 expression $(p > 0.05 \ n = 6)$.
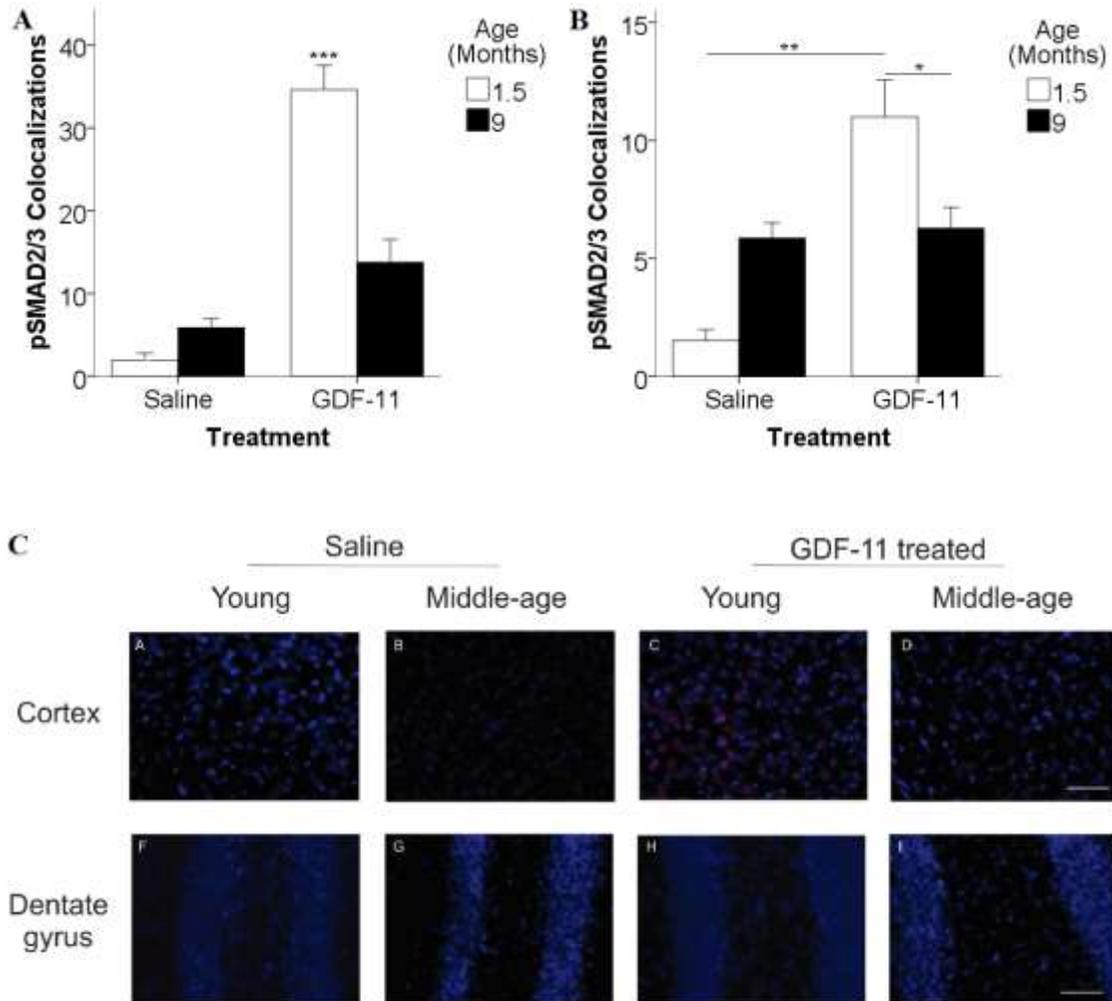
Figure 8: pSMAD2/3 expression in the cortex and dentate gyrus. A) Quantitative analysis of co-localizations in the cortex shows a significant increase in pSMAD2/3 levels in 1.5-months old mice treated with rGDF-11 compared to all other groups. B) Co-localization counts in the dentate gyrus are lower in number compared to the cortex, but show similar trends, with 1.5-months old mice treated with rGDF-11 significantly higher in number than other groups. C) Representative image of the cortex and dentate gyrus, at 20x magnification, of pSMAD2/3 (red) over DAPI (blue). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, Bonferroni post-hoc pairwise comparison.

pAkt protein expression is significantly higher in the brains of treated young mice

As pSMAD2/3 did not appear promising as a possible mechanism to explain the functional improvements in middle aged mice, another effector of GDF-11 that was not related to the pSMAD2/3 pathway was evaluated. Phosphorylated Akt (pAkt) is another important downstream effector of GDF-11 (Egermann and Glass, 2014) known for a plethora of effects, including neuronal survival (Noshita et al, 2002). Western blot of whole brain lysate was carried out, and the results are shown in Figure 9. Two-way ANOVA showed an interaction between age and treatment ($F_{(1,16)} = 7.9$, $p < 0.05$, n = 5). Bonferonni post-hoc test showed significantly higher pAkt protein levels in GDF-11 treated young mice ($p < 0.01$, n = 5). This result once again confirms that the increased GDF-11 levels detected previously in the brains of young GDF-11 treated mice is activating the GDF-11 cascades, but still did not provide a mechanism for the behavioral improvement observed in middle-age mice.
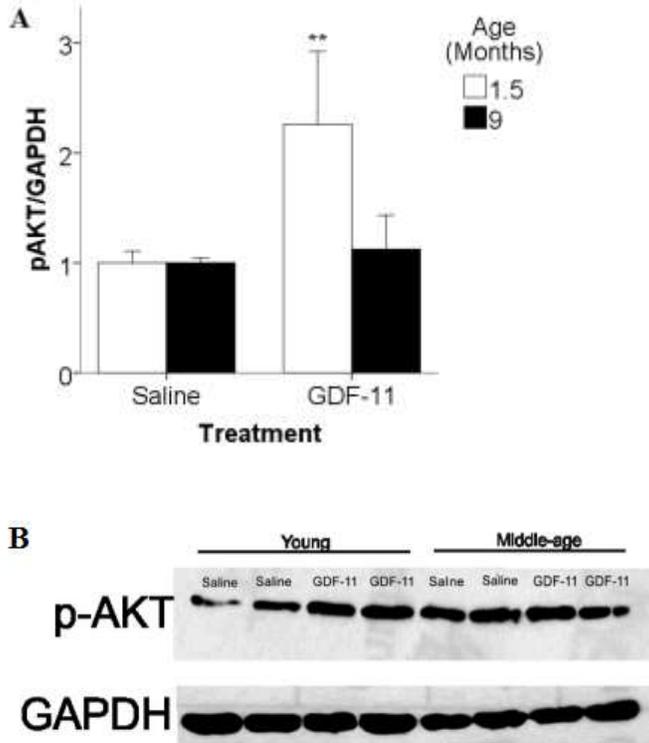
Figure 9: pAkt protein expression in whole brain lysate. A) pAkt western blot data after normalization via GAPDH. 1.5-months old mice treated with rGDF-11 showed significantly higher pAkt expression than other groups. B) Representative image of pAkt western blot. **$p < 0.01$, Bonferroni post-hoc pairwise comparison.

Increased PH3 and DCX expression in brain regions for treated middle-aged mice

GDF-11 was previously shown to be effective at increasing both neurogenesis and odor detection tests in aged mice (Katsimpardi et al, 2014). As the DG is one of the few sites for adult neurogenesis in both mice and humans, we used immunofluorescence to detect for neurogenesis markers in the DG of treated mice, results of which are shown in Figure 8. Two previously validated neurogenesis markers were used: DCX and PH3 (Rao and Shetty, 2004, Komitova et al, 2005). Independent t-test of DCX levels in the DG for young and middle-age mice treated with GDF-11 showed a significantly higher expression in middle-age brains ($t_{(7)} = 4.74$, $p < 0.01$, n = 4-5) (Figure 10A). Similarly, PH3 expression was significantly higher for middle-age mice treated with GDF-11 compared to young mice ($t_{(8)}=4.76$, $p < 0.01$, n = 5) (Figure 10B). This suggested that GDF-11 promoted an increase in neurogenesis that was correlated with the improved visual memory of middle-age mice treated with GDF-11, and could present a possible mechanism for the behavioral improvement. There are several reasons why this rapid induction of neurogenesis could be observed, which could be related to the potential impact of GDF-11 on neurotrophic support in the brain (Ge et al, 2005, Lu et al 2005, Bouchard and Villeda, 2015). Indeed, GDF-11 had been previously shown to target expression of brain derived neurotrophic factor (BDNF) (Ge et al, 2005), among others.
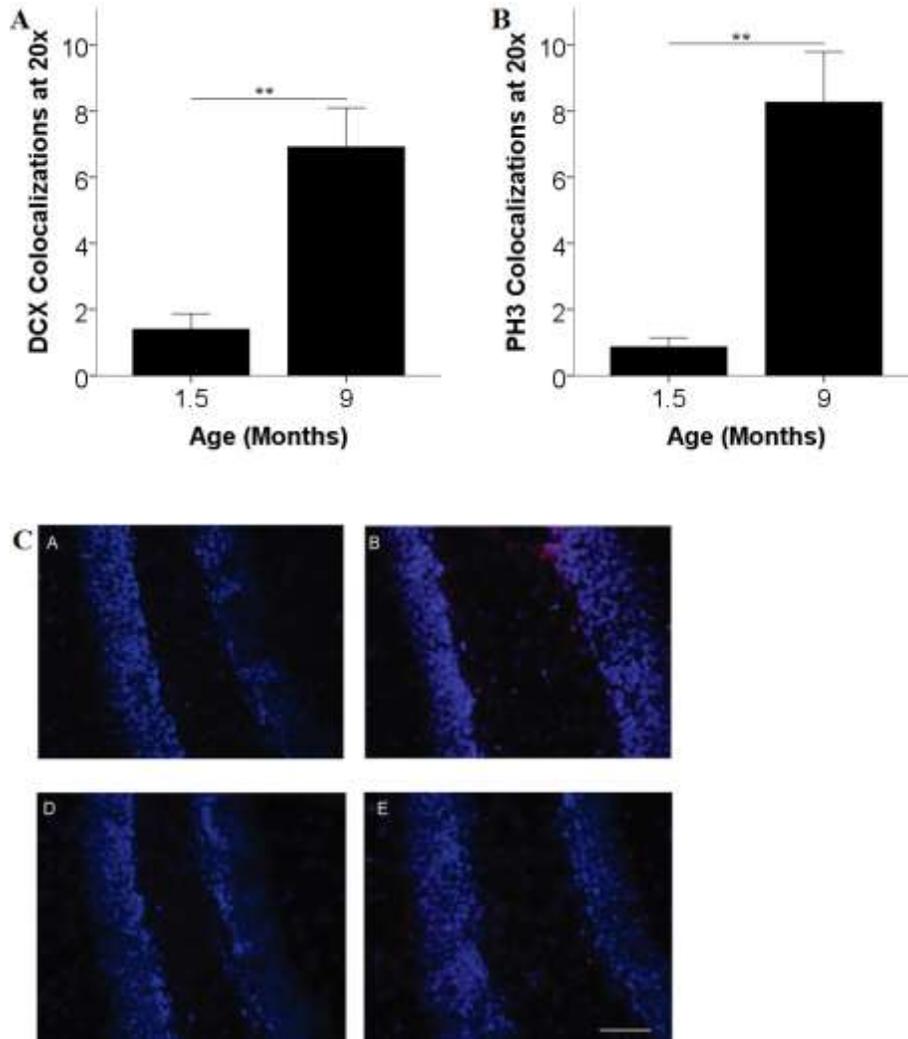
Figure 10: Neurogenesis marker expression in the dentate gyrus of rGDF-11 treated mice. A) Quantification of DCX co-localization. 9-months old mice show significant increase in expression. B) Quantification of PH3 co-localization. 9-months old mice show significant increase in expression. C) Representative images of DCX expression (red) over DAPI (blue) in 1.5-months old mice (a) and 9-months old mice (b), and PH3 expression (red) over DAPI (blue) in 1.5-months old mice (d) and 9-months old mice (e). **$p < 0.01$, Bonferroni post-hoc pairwise comparison.

Discussion

The data presented in this thesis demonstrate that a single intraperitoneal injection of 0.1mg/kg rGDF-11 was sufficient to counteract the age-related decline in short term memory in middle-aged mice, showing a dramatic improvement in visual memory. One injection of rGDF-11 resulted in a rapid enhancement in neurogenesis in the middle-aged mouse brain, suggesting a possible mechanism of action for the behavioral improvement. This initiation of neurogenesis could reflect a GDF-11 induced enhancement in neurotrophic support in the brain, which could account for the rapid behavioural improvement observed. Indeed, previous research has shown that several neurotrophic factors are targets of GDF-11 (Ge et al, 2005, Lu et al, 2005, Bouchard and Villeda, 2015).

Baseline behavior experiments for young and middle-aged C57Bl/6 mice.

Baseline testing of 1.5 months-old (young) and 9-months old (middle-aged) showed a significant decline in performance in the visual short term memory, using the NOR task. This decline is similar in humans in the early middle-age years, where an observable decline in short-term memory task performance becomes statistically relevant (Salthouse, 2009). Whether this deficit is due to decline in processing speed or other mechanisms, it is clear that visual memory generally declines with age and can be evident quite early in the middle aged years. This loss in visual memory could be an initial

indicator of the initiation of age-related cognitive decline and could be a precursor to cognitive dysfunction seen in more advanced old age.

At 9-months old, the spatial memory-testing Y-maze task did not show any significant age-related decline. It has been previously shown that a significant decline to spatial memory was present between young and middle-aged mice, however the middle-aged mice had a more diverse age group of 9-12-months of age (Gower and Lamberty, 1993). Another likely contributing factor for this difference in results was due to the difference in behavior task. The previous work used a Morris Water Maze and based its results on a training regimen, while the current work used a Y-maze test that looked only at very short-term memory (seconds to minutes) for mice at one intersection, which is a less difficult memory task.

Behavioral impact of rGDF-11 treatment.

The data collected toward this thesis showed significant improvement in performance of the NOR task for the middle-aged mice treated with rGDF-11 compared to middle-aged mice treated with a control saline 24 hours after treatment. Another significance was also detected between young mice treated with rGDF-11 and middle-aged control mice. No increase in performance was observed between young mice treated with rGDF-11 compared to the young mice control group. Surprisingly, when compared to the baseline experiments, there was no significant change in NOR task performance between control groups of different ages, but statistically the p-value for the Bonferonni test between the two groups was so incredibly close to significance, it was likely that

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significance would have been reached should there have been a greater number of test animals per group.

These results suggest that a singular injection of rGDF-11 was sufficient to significantly improve visual memory in mice, but when compared within their own age groups, there is only significant improvement in older mice, after cognitive decline had begun.

GDF-11 and its downstream cascade.

In order to better understand the mechanisms behind the behavior effects of rGDF-11, this study investigated the protein levels of GDF-11 in the brain and its direct downstream cascade in the dentate gyrus and cortex, two regions known to be involved in visual memory. This study showed a significant increase in GDF-8+11 protein levels in the brain of young mice treated with rGDF-11 that was not replicated in the middle-aged mice. Due to the non-specificity of the antibody used to label GDF-11, it was necessary to observe protein levels of its family member GDF-8, and this study demonstrated that in the dentate gyrus of the hippocampus and in the cortex, GDF-8 protein expression was consistent between rGDF-11 treated mice in both age groups in both regions. This then suggests that the increase in protein level detected was due to GDF-11, and not GDF-8.

In a similar trend, protein expression of pSMAD2/3, the well characterized downstream effector of GDF-11(Katsimpardi et al, 2014, Zhang et al, 2016), was significantly elevated in young mice treated with rGDF-11 but not middle-aged mice. Similarly, when comparing within age groups, rGDF-11 treated young mice show

significant elevation compared to the young control group, while no significant differences were observed between the middle-aged rGDF-11 treated mice and control group. This trend was again reflected when determining the brain protein levels of pAkt, a known downstream effector of GDF-11 involved in cell survival and proliferation (McPherron, 2010, Brun and Rudnicki, 2015), with young mice treated with rGDF-11 having significantly higher protein levels of pAkt than middle-aged mice.

As pSMAD2/3 is the most well-known secondary messenger of GDF-11 and is believed to be the primary known method GDF-11 uses to affect cellular changes via DNA methylation (Schmierer and Hill, 2007), these findings suggest that the direct downstream cascade of GDF-11 was unlikely the main mechanism behind the recovery of visual memory functions to pre-decline levels in middle-aged mice treated with rGDF-11. In fact, GDF-11 protein levels seems to indicate that GDF-11 levels in middle-aged mice brains were not changed at all compared to control mice. One possible explanation for this is an age-related change to the composition of the blood-brain barrier (BBB) that is preventing GDF-11 from entering in middle-aged mice, and rodents do have tighter BBBs than most model organisms (Bradbury, 1979). It is, however, too early to come to such a conclusion- GDF-11, similar to GDF-8, do not exist in active form in the bloodstream, but rather inactivated by bound proteins such as the protease inhibitor GDF-associated serum protein-1 (GASP-1) (Hill et al, 2008), as well as its own pro-peptide (Annes et al, 2003, Schmierer and Hill, 2007, Hill et al, 2008, McPherron, 2010). As the rGDF-11 treated mice were given one single injection of 0.1mg/kg rGDF-11, which is known to be elevated in the bloodstream consistently at 24 hours after injection but not after (Loffredo et al, 2013), a possible explanation for the seemingly absent GDF-11 in

the brains of middle-aged mice was due to consumption, while the elevated GDF-11 found in young treated mice are inactive, bound by GASP-1/2 and other inhibitory molecules, unable to bind to its receptors.

Neurogenesis in rGDF-11 treated mice.

Aged mice undergoing rGDF-11 treatment were previously shown to have significantly increased neurogenesis in the sub-ventricular zone and angiogenesis throughout the brain (Katsimpardi et al, 2014). As the DG also undergoes significant adult neurogenesis (Cameron and McKay, 2001) that has been linked to memory formation (Gould et al, 1999, Snyder et al, 2005) we quantified neurogenesis in the DG for all treatment groups. DCX and PH3 are two neurogenesis markers that bind to new neurons and cells leaving the S phase of mitosis, respectively, to obtain a more accurate view of neurogenesis levels and ensure higher confidence in the results. Both DCX and PH3 expression was demonstrated to be significantly greater in middle-aged mice treated with rGDF-11 when compared to young mice treated with rGDF-11, a noticeable result considering adult neurogenesis should be equal if not slowing down when comparing 9-months old middle-aged mice to young 1.5-months old mice (Dutta and Sengupta, 2015). While the correlative trends between neurogenesis and NOR performance between treatment groups is promising, unfortunately the earliest neurogenesis in the DG has been positively linked to improved memory is 4 days after neurogenesis (Snyder et al, 2005), which disqualifies it from being a direct mechanism contributing to the cognitive improvement. However, it was shown that hippocampal related learning and memory

formation improves adult neurogenesis in the DG (Gould et al, 1999, Snyder et al, 2005),

thus this observed increase in neurogenesis post-treatment in middle-aged mice is likely

beneficial to the cognitive functions of the treated mice. Furthermore, it is possible that

the direct mechanisms responsible for the cognitive improvements are also responsible

for the increase in expression of early neurogenesis markers, and are regulators of

neurogenesis and involved in neuroplasticity. Neurotrophic factors are a family of

molecules that fit all the above criteria- known to support neuronal survival, involved in

neuron differentiation and maturation, and play a role in synaptic plasticity (Ip and

Yancopoulous, 1996, Lo, 1995, Frostick et al, 1998).

One possible candidate is Brain Derived Neurotrophic Factor (BDNF), which is

known to be involved in neurogenesis, hippocampal learning, and has been suggested to

have a co-regulatory relationship with GDF-11 and the TGF-β superfamily (Hall et al,

2000, Lu et al, 2005, Lu et al, 2008, Bickford et al, 2015). Another possible contributor is

Nerve Growth Factor (NGF), the "archetypical neurotrophin" (Lo, 1995) that is known to

have functions directly regulated by GDF-11, such as neuro-differentiation (Ge et al,

2005). NGF expression decreases with age in the brain, and NGF treatment has been

shown to have similar cognitive rejuvenation effects as GDF-11 in rodent models

(Fischer et al, 1991, Terry Jr et al, 2011). Finally, this paper suggests Glial cell line-

Derived Neurotrophic Factor (GDNF) as a third possible target of GDF-11. GDNF is a

neurotrophic factor family that is related to the TGF-β super-family (Frostick et al, 1998),

contributing to the development of the peripheral nervous system (Baloh et al, 2000).

GDNF receptor knock-out mice were observed to have impaired memory and behavioral

flexibility (Voikar et al, 2004), and GDNF treatment of aged rats through a transgene

inserted into the astrocyte results in significant improvements in memory and learning

(Pertusa et al, 2008). While the relationship between neurotrophic factors and GDF-11 is

currently poorly understood, the known functions of these molecules make for promising

areas of further research.

Conclusion

To our knowledge, this is the first set of data to show that rGDF-11 is able to restore age-related cognitive decline in middle-aged mice. The results suggest that a single dose of rGDF-11 was sufficient to alleviate the decline of visual memory in middle-aged, 9-months old male C57Bl/6 mice. Increased neurogenesis in the dentate gyrus of 9-months old treated mice was detected 30 hours after injection with rGDF-11. Early indicators of enhanced neurogenesis also presents a possible clue to the mechanisms involved in the restoration of cognitive function, mediated by neuroplasticity and enhanced neurotrophic support.

References

Aigner L and Bodgahn U. TGF-beta in neural stem cells and in tumors of the central nervous system. Cell and Tissue Research. 331(1):225-241. 2008.

Andersen RE and Lim DA. An ingredient for the elixir of youth. Cell Research. 24:1381-1382. 2014.

Andersson O, Reissmann E and Ibanez CF. Growth differentiation factor 11 signals through the transforming growth factor-β receptor ALK5 to regionalize the anterior-posterior axis. EMBO Reports. 7(8):831-837. 2006.

Andrews-Hanna JR, Snyder AZ, Vincent JL, Lustig C, Head D, Raichle ME and Buckner RL. Disruption of large-scale brain systems in advanced aging. Neuron. 56(5):924-935. 2007.

Annes J, Munger J and Rifkin D. Making sense of latent TGFβ activation. Journal of Cell Science. 116:217-224. 2003.

Antunes M and Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cognitive Processing. 13(2):93-110. 2012.

Ardila A. Normal aging increases cognitive heterogeneity: analysis of dispersion in WAIS-III scores across age. Archives of Clinical Neuropsychology. 22:1003-1011. 2007.

Balemans W and van Hul W. Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Developmental Biology. 250(2):231-250. 2002.

Baloh R, Enomoto H, Johnson Jr E and Milbrandt J. The GDNF family ligands and

    receptors – implications for neural development. Current Opinion in

    Neurobiology. 10(1):103-110. 2000.

Bevins RA and Besheer J. Object recognition in rats and mice: a one-trial non-matching-

    to-sample learning task to study 'recognition memory'. Nature Protocols.

    1(3):1306-1311. 2006.

Bickford P, Kaneko Y, Grimmig B, Pappas C, Small B, Sanberg C, Sanberg P, Tan J and

    Shytle R. Nutraceutical intervention reverses the negative effects of blood from

    aged rats on stem cells. Age. 37:103. 2015.

Bishop NA, Lu T and Yankner BA. Neural mechanisms of ageing and cognitive decline.

    Nature. 464:529-535. 2010.

Bjornsti MA and Houghton PJ. The tor pathway: a target for cancer therapy. Nature

    Reviews: Cancer. 4:335-348. 2004.

Bouchard J and Villeda S. Aging and brain rejuvenation as systemic events. Journal of

    Neurochemistry. 132(1):5-19. 2015.

Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS and Schreiber SL. A

    mammalian protein targeted by G1-arresting rapamycin-receptor complex.

    Nature. 369(6483):756-758. 1994.

Brun C and Rudnicki M. GDF11 and the Mythical Fountain of Youth. Cell Metabolism.

    22(1):54-56. 2015.

Buckwalter M, Yamane, M, Coleman B, Ormerod B, Chin J, Palmer T, and Wyss-Coray T. Chronically increased transforming growth factor-β1 strongly inhibits hippocampal neurogenesis in aged mice. American Journal of Pathology. 169(1):154-164. 2006.

Carrel A. Concerning Visceral Organisms. Journal of Experimental Medicine. 18(2):155-161. 1913.

Cell Signaling. TGF-β signaling. Cell Signaling Technology. 2014.

Conboy M, Conboy I, and Rando T. Heterochronic parabiosis: historical perspective and methodological considerations for studies of aging and longevity. Aging Cell. 12(3):525-530. 2013.

Conidi A, Cazzola S, Beets K, Coddens K, Collart C, Cornelis F, Cox L, Joke D, Dobreva MP, Dries R, Esguerra C, Francis A, Ibrahimi A, Kroes R, Lesage F, Maas E, Moya I, Pereira PN, Stappers E, Stryjewska A, van den Berghe V, Vermeire L, Verstappen G, Seuntjens E, Umans L, Zwijsen A and Huylebroeck D. Few Smad proteins and many Smad-interacting proteins yield multiple functions and action modes in TGFbeta/BMP signaling in vivo. Cytokine & Growth Factor Reviews. 22(5-6): 287-300. 2011.

Deary IJ, Corley J, Gow AJ, Harris SE, Houlihan LM, Marioni RE, Penke L, Rafnsson SB and Starr JM. Age-associated cognitive decline. British Medical Bulletin. 92:135-152. 2009.

Dutta S and Sengupta P. Men and mice: relating their ages. Life Sciences. In press. 2015.

52

Egerman MA, Cadena SM, Gilbert JA, Meyer A, Nelson HN, Swalley SE, Mallozzi C, Jacobi C, Jennings LL, Clay I, Ma GS, Brachat S, Lach-Trifilieff E, Shavlakadze T, Trendelenburg AU, Brack AS and Glass D. GDF11 increases with age and inhibits skeletal muscle regeneration. Cell Metabolism. 22(1):164-174. 2015.

Esquela A and Lee S. Regulation of metanephric kidney development by growth-differentiation factor 11. Developmental Biology. 15;257(2):356-70.

Flanders K, Ludecke G, Engels S, Cissel D, Roberts A, Kondaiah P, Lafyatis R, Sporn M and Unsicker K. Localization and actions of transforming growth factor-βs in the embryonic nervous system. Development. 113:183-191. 1991.

Fischer W, Bjorklund A, Chen K and Gage F. NGF improves spatial memory in aged rodents as a function of age. Journal of Neuroscience. 11(7):1889-1906.

Fotenos AF, Snyder AZ, Girton LE, Morris JC and Buckner RL. Normative estimates of cross-sectional and longitudinal brain volume decline in aging and AD. Neurology. 64(6):1032-1039. 2005.

Frostick S, Yin Q and Kemp G. Schwann Cells, Neurotrophic Factors, and Peripheral Nerve Regeneration. Microsurgery. 18:397-405.1998.

Gamer LW, Wolfman NM, Celeste AJ, Hattersley G, Hewick R and Rosen V. A novel BMP expressed in developing mouse limb, spinal cord, and tail bud is a potent mesoderm inducer in *Xenopus* embryos. Developmental Biology. 208(1):222-232. 1999.

Ge G, Hopkins DR, Ho WB and Greenspan DS. GDF11 forms a bone morphogenetic

protein 1- activated latent complex that can modulate nerve growth factor-

induced differentiation of PC12 cells. Molecular Cell Biology. 25(14):5846-

5858. 2005.

George O, Parducz A, Dupret D, Kharouby M, le Moal M, Piazza PV and Mayo W.

Smad-dependent alterations of PPT cholinergic neurons as a pathophysiological

mechanism of age-related sleep-dependent memory impairments. Neurobiology

of Aging. 27(12):1848-1858. 2006.

Goraksha-Hicks P and Rathmell JC. TGF-β: a new role for an old AktTOR.

Developmental Cell. 17(1):6-8. 2010.

Gould E, Beylin A, Tanapat P, Reeves A and Shors T. Learning enhances adult

neurogenesis in the hippocampal function. Nature Neuroscience. 2:260-265.

1999.

Gower AJ and Lamberty Y. The aged mouse as a model of cognitive decline with special

emphasis on studies in NMRI mice. Behavioural Brain Research. 57:163-173.

1993.

Hall J, Tomas K and Everitt B. Rapid and selective induction of BDNF expression in the

hippocampus during contextual learning. Nature Neuroscience. 3:533-535. 2000.

Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL,

Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E and

Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 460:392-395. 2009.

Herpin A, Lelong C and Favrel P. Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. Developmental and Comparative Immunology. 28(5):461-485. 2004.

Hill J, Qiu Y, Hewick R and Wolfman N. Regulation of Myostatin in Vivo by Growth and Differentiation Factor-Associated Serum Protein-1: A Novel Protein with Protease Inhibitor and Follistatin Domains. Molecular Endocrinology. 2008.

Horbelt D, Denkis A and Knaus P. A portrait of transforming growth factor β superfamily signalling: background matters. The International Journal of Biochemistry & Cell Biology. 44(3): 469-474. 2011.

Huang Z, Chen X and Chen D. Myostatin: a novel insight into its role in metabolism, signal pathways, and expression regulation. Cell Signal. 23(9):1441-1446. 2011.

Huminiecki L, Goldovsky L, Freilich S, Moustakas A, Ouzounis C and Heldin CH. Emergence, development and diversification of the TGF-β signalling pathway within the animal kingdom. BMC Evolutionary Biology. 9:28. 2009.

Huse M, Muir TW, Xu L, Chen YG, Kuriyan J and Massague J. The TGFβ receptor activation process: an inhibitor- to substrate-binding switch. Molecular Cell. 8(3):671-682. 2001.

Ikushima H and Miyazono K. TGFbeta signalling: a complex web in cancer progression. Nature Reviews: Cancer. 10(6):415-424. 2010.

55

Ip N and Yancopoulos G. The Neurotrophins and CNTF: Two Families of Collaborative
Neurotrophic Factors. Annual Reviews Neuroscience. 19:491-515. 1996.

Kamran P, Sereti KI, Zhao P, Ali S, Weissman I, and Ardehali R. Parabiosis in Mice: A
Detailed Protocol. Journal of Visualized Experiments. 80:50556. 2013.

Katsimpardi L, Litterman NK, Schein PA, Miller CM, Loffredo FS, Wojtkiewicz GR,
Chen JW, Lee RT, Wagers AJ and Rubin LL. Vascular and neurogenic
rejuvenation of the aging mouse brain by young systemic factors. Science.
344:630-633. 2014.

Kennard JA and Woodruff-Pak D. Age sensitivity of behavioral tests and brain substrates
of normal aging in mice. Frontiers in Aging Neuroscience. 3(9):1-22. 2011.

Kral VA. Senescent forgetfulness: benign and malignant. Canadian Medical Association
Journal. 86(6):257-260. 1962.

Lee Y and Lee S. Regulation of GDF-11 and myostatin activity by GASP-1 and GASP-2.
Proceedings of the National Academy of Sciences of the United States of
America. 110(39):E3713-E3722. 2013.

Lo D. Neurtrophic Factors and Synaptic Plasticity. Neuron. 15:979-981. 1995.

Loffredo FS, Steinhauser ML, Jay SM, Gannon J, Pancoast JR, Yalamanchi P, Sinha M,
Dall'Osso C, Khong D, Shadrach JL, Miller CM, Singer BS, Stewart A,
Psychogios N, Gerszten RE, Hartigan AJ, Kim MJ, Serwold T, Wagers AJ and
Lee RT. Growth differentiation factor 11 is a circulating factor that reverses age-
related cardiac hypertrophy. Cell. 153(4):828-839. 2013.

Lu J, Wu Y, Sousa N and Almeida O. SMAD pathway mediation of BDNF and TGFβ2

    regulation of proliferation and differentiation of hippocampal granule neurons.

    Development. 132:3231-3242. 2005.

Lu Y, Christian K and Lu B. BDNF: A key regulator for protein synthesis-dependent

    LTP and long-term memory? Neurobiology of Learning and Memory.

    89(3):312-323. 2008.

Massague J. TGFβ signalling in context. Nature Reviews: Molecular Cell Biology.

    13:616-630. 2012.

McPherron A. Metabolic Functions of Myostatin and GDF11. Immunology Endocrine &

    Metabolic Agents. 10(4):217-231. 2010.

Mira H, Andreu Z, Suh H, Lie DC, Jessberger S, Consiglio A, San Emeterio J, Hortiguela

    R, Margues-Torrejon MA, Nakashima K, Colak D, Gotz M, Farinas I and Gage

    FH. Signaling through BMPR-IA regulates quiescence and long-term activity of

    neural stem cells in the adult hippocampus. Cell: Stem Cell. 7(1):78-89. 2010.

Miyazono K. Positive and negative regulation of TGF-β signaling. Journal of Cell

    Science. 113:1101-1109. 2000.

Miyazono K. Transforming growth factor-beta signaling in epithelial-mesenchymal

    transition and progression of cancer. Proceedings of the Japan Academy, Series

    B, Physical and Biological Sciences. 85(8):314-323. 2009.

Nakashima M, Tovono T, Akamine A and Joyner A. Expression of growth/differentiation factor 11, a new member of the BMP/TGFbeta superfamily during mouse embryogenesis. Mechanisms of development. 80(2):185-189. 1999.

Ohl F, Roedel A, Binder E and Holsboer F. Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice. European Journal of Neuroscience. 17(1):128-136. 2003.

Pang K, Ryan JF, Baxevanis AD and Martindale MQ. Evolution of the TGF-β signaling pathway and its potential role in the ctenophore, *Mnemiopsis leidyi*. Public Library of Science. 6(9):e24152. 2011.

Park DC and Schwarz N. Cognitive aging: a primer. Psychology Press. 2012.

Pertusa M, Garcia-Matas, Mammeri H, Adell A, Rodrigo T, Mallet J, Cristofol R, Sarkis C and Sanfeliu C. Expression of GDNF transgene in astrocytes improves cognitive deficits in aged rats. Neurobiology of Aging. 29(9):1366-1379. 2008.

Poggioli T, Vujic A, Yang P, Macias-Trevino C, Uygur AN, Loffredo FS, Pancoast JR, Cho M, Goldstein J, Tandias RM, Gonzalez E, Walker RG, Thompson TB, Wagers AJ, Fong YW and Lee RT. Circulating growth differentiation factor 11/8 levels decline with age. Circulation Research. Epub: 115.307521. 2015.

Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ and Rubinsztein DC. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nature Genetics. 36(6):585-595. 2004.

Rodgers BD and Eldridge JA. Reduced circulating GDF11 is unlikely responsible for
age-dependent changes in mouse heart, muscle and brain. Endocrinology.
156(11):3885-3888. 2015.

Rowe JW and Kahn RL. Successful aging. The Gerontologist. 37(4):433-440. 1997.

Ryan JJ, Sattler JM and Lopez SJ. Age effects on Wechsler Adult Intelligence Scale-III
subtests. Archives of Clinical Neuropsychology. 15:311-317. 2000.

Sabo JK, Kilpatrick TJ and Cate HS. Effects of bone morphogenic proteins on neural
precursor cells and regulation during central nervous system injury.
Neurosignals. 17(4):255-264. 2009.

Salthouse TA. When does age-related cognitive decline begin? Neurobiology of Aging.
30:507-514. 2009.

Schmierer B and Hill C. TGFβ-SMAD signal transduction: molecular specificity and
functional flexibility. Nature Reviews Molecular Cell Biology. 8:970-982. 2007.

Schroeder DH and Salthouse TA. Age-related effects on cognition between 20 and 50
years of age. Personality and Individual Differences. 36:394-404. 2004.

Shi Y and Massague J. Mechanisms of TGF-β signaling from cell membrane to the
nucleus. Cell. 113(6):685-700. 2003.

Singh A and Morris RJ. The yin and yang of bone morphogenetic proteins in cancer.
Cytokine Growth Factor Reviews. 21(4):299-313. 2010.

Smith SC, Zhang X, Zhang X, Gross P, Starosta T, Mohsin S, Franti M, Gupta P, Hayes D, Myzithras M, Kahn J, Tanner J, Weldon SM, Khalil A, Guo X, Sabri A, Chen X, MacDonnell S and Houser SR. GDF11 does not rescue aging-related pathological hypertrophy. Circulation Research. 117(11):926-932. 2015.

Snyder J, Hong N, McDonald R and Wojtowicz J. A role for adult neurogenesis in spatial long-term memory. Neuroscience. 30(4):843-852. 2005.

StatCan. Portrait of Canada's Labour Force. Statistics Canada. 2011.

StatCan. Canada's population estimates: age and sex, July 1, 2015. Statistics Canada. 2015.

Terry Jr, A, Kutiyanawalla A and Pillai A. Age-dependent alterations in nerve growth factor (NGF)-related proteins, sortilin, and learning and memory in rats. Physiology & Behavior. 102(2):149-157. 2011.

Villeda SA, Plambeck KE, Middeldorp J, Castellano JM, Mosher KI, Luo J, Smith LK, Bieri G, Lin K, Berdnik D, Wabl R, Udeochu J, Wheatley EG, Zou B, Simmons DA, Xie XS, Longo FM and Wyss-Coray T. Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. Nature Medicine. 20:659-663. 2014.

Voikar V, Rossi J, Rauvala H and Airaksinen M. Impaired behavioural flexibility and memory in mice lacking GDNF family receptor α2. European Journal of Neuroscience. 20(1):308-312. 2004.

60

Walker R, Poggioli T, Katsimpardi L, Buchanan S, Oh J, Wattrus JS, Heidecker B, Fong
Y, Rubin L, Ganz P, Thompson T, Wagers A, and Lee R. Biochemistry and
Biology of GDF11 and Myostatin: Similarities, Differences and Questions for
Future Investigation. Circulation Research. DOI:
10.1161/CIRCRESAHA.116.308391. 2016.

Wu HH, Ivkovic S, Murray RC, Jaramillo S, Lyons KM, Johnson JE and Calof AL.
Autoregulation of neurogenesis by GDF11. Neuron. 37(2):197-207. 2003.

Wu X, Shi W and Cao X. Multiplicity of BMP Signaling in Skeletal Development.
Annals of the New York Academy of Sciences. 1116:29-49. 2007.

Yankner BA, Lu T and Loerch P. The aging brain. Annual Review of Pathology:
Mechanisms of Disease. 3:41-66. 2008.

Zhang Y. Non-Smad pathways of TGF-β signaling. Cell research. 19:128-139. 2009.

Zhang Y, Cheng F, Du X, Gao J, Li N, Li S and Dong de L. GDF11/BMP11 activates
both smad1/5/8 and smad2/3 signals but shows no significant effect on
proliferation and migration of human umbilical vein endothelial cells.
Oncotarget. 7(11):12063-12074. 2016.

Zhou Y, Jiang Z, Harris E, Reeves J, Chen X and Pazdro R. Circulating concentrations of
growth differentiation factor 11 are heritable and correlate with life span.
Journals of Gerontology. Doi: 10.1093/Gerona/glv308. 2016.

Zhu H, Kavsak P, Abdollah S, Wrana JL and Thomsen GH. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature. 400(6745):687- 1999.