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Srijita Nandy April 6, 2022

AAV-GRN subunit injections can rescue FTD disease phenotypes

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Abstract

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By Srijita Nandy

Frontotemporal dementia (FTD) is the most common cause of dementia in those under the age of 60. FTD is characterized by the neurodegeneration of the frontal and temporal lobes and can cause behavioral disinhibition, apathy, or difficulty speaking or understanding language. There is no treatment for the underlying cause of FTD. One cause of FTD is heterozygous mutations of the granulin (*GRN)* gene which leads to haploinsufficiency of the progranulin (PGRN) protein and its granulin subunits. While the precise function of PGRN and granulins are unknown, prior research has shown that disease phenotypes of FTD-like mice can be rescued using adeno-associated virus (AAV) encoding PGRN. However, AAV-PGRN may not be a viable clinical therapy because PGRN is a pleiotropic protein and administration of AAV-PGRN to the brain is challenging. Thus, granulin subunits have become proteins of interest in the disease pathology of FTD-*GRN*. While the full function of granulin subunits in the disease pathology of FTD-*GRN* is unknown, prior research has hypothesized that granulin subunits may be toxic or harmful. This study aims to understand if AAV constructs of granulin subunits will exacerbate or rescue disease phenotypes in the hippocampus of FTD-like mice. This study found the first evidence that granulin subunits can rescue disease phenotypes in an *in vivo* model of FTD.

AAV-GRN subunit injections are able to rescue FTD disease phenotypes

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Introduction

Frontotemporal dementia (FTD)

Frontotemporal dementia (FTD) is the most common cause of dementia in people under the age of 60 (Bang et al., 2015), and it is the second most common form of dementia overall, only behind Alzheimer's disease (Leroy et al., 2021). The neuropathology that underlies FTD, termed frontotemporal lobar degeneration (FTLD) is characterized by progressive degeneration of both the temporal and the frontal lobes of the brain (Young et al., 2018). Given the extent of degeneration, FTD is a fatal diagnosis. It is currently unclear to what extent certain gene and environmental factors contribute to the clinical presentation of FTD. Further, as there are no good biomarker tests for FTD, an FTD diagnosis currently depends on the symptoms presented by the patient (Musa et al., 2020). As such, many patients are not diagnosed with FTD for ~4 years from symptom onset (Diehl and Kurz, 2002; Benussi et al., 2015; Swift et al., 2021). Currently, an FTD diagnosis depends on the symptoms presented by the patient (Musa et al., 2020). This, however, is not always reliable because FTD patients can experience a wide range of symptoms, many of which correlate with other neurodegenerative diseases as well as multiple psychiatric disorders (Musa et al., 2020). The variability of FTD symptom presentation can oftentimes lead to patients being misdiagnosed; many people who suffer from FTD are diagnosed with bipolar disorder, depression, or mania (Beber and Chaves, 2013), and only after further disease progress or a post-mortem analysis, is the FTD uncovered. Because clinical diagnosis of FTD is so challenging, the only way to definitively diagnose is to perform a

post-mortem pathological study that identifies pathological inclusions known to cause FTD (Irwin, et al., 2018).

Pathological inclusions are formed from aggregates of misfolded proteins and are widely proposed to be a major contributor to the neuronal dysfunction and eventual neurodegeneration seen in FTD patients. The major components of these pathological inclusions found in FTD patients help to distinguish between the four subtypes of FTD. The four subtypes include FTD-Tau, FTD-TDP, FTD-FUS, and FTD-UPS which are characterized by pathological inclusions predominantly consisting of Tau protein deposits, TDP-43 deposits, FUS deposits, and unknown protein deposits respectively (Goedert, et al., 2012). FTD-Tau and FTD-TDP are the most common presentations of the disease (Goedert, et al., 2012).

While definitive diagnosis of FTD is based on the presence of pathological inclusions postmortem, currently in presenting patients, physicians primarily depend on identifying the two main clinical presentations of FTD: behavioral-variant FTD (bvFTD) and primary progressive aphasia (PPA) (Ljubenkov and Miller, 2016). PPA is the less common form of FTD (Bang et al., 2015; Erkkinen et al., 2018; Hogan et al., 2016; Johnson et al., 2005). Patients who are diagnosed with PPA experience a form of FTD characterized by difficulty with the pronunciation of words as well as difficulty with repeating messages verbatim (Marshall et al., 2018). The majority of FTD cases are clinically classified bvFTD, where patients experience symptoms such as behavioral disinhibition, apathy, and socially inappropriate behavior (Bang et al., 2015; Erkkinen et al., 2018; Hogan et al., 2016; Johnson et al., 2015). The majority of patients with FTD caused by *GRN* mutations present with bvFTD. Currently, the only available treatments

for FTD patients help to manage the clinical symptoms, but do not address the underlying disease (Root, et al., 2021). Although it can be highly variable with reported cases of patients who have been diagnosed with FTD as early as 35 years of age, the average onset age for FTD is around 50 years of age (Gossye, et al., 2019). With no treatment, this means that fairly early in life, the quality of life for FTD patients deteriorates rapidly as they experience drastic personality changes, apathy, compulsive behavior, and some memory loss (Gossye, et al., 2019) until the patient death approximately seven to thirteen years after the time of diagnosis (Onyike, 2011). Unfortunately, treatment options for FTD patients and their families remain limited. There are currently no treatments available that can slow or stop the spread of FTD pathology, limit the degeneration of neurons, and improve outcomes for individuals. Therefore, developing more effective treatments is a critical clinical need.

While the overall cause of FTD remains unknown, mutations identified in cases of the disease can provide insight into the etiology. FTD can be hereditary. In fact, approximately 30% of individuals diagnosed with FTD have a family history of the disease (Greaves and Rohrer, 2019), however, the majority of patients diagnosed with FTD do not have a family history of neurodegenerative disease at all (Rohrer et al., 2009). Mutations in several autosomal genes have been found to cause FTD (Olszewska, et al., 2016; Kao et al., 2017). Mutations of the granulin gene (*GRN*), which encodes the protein progranulin (PGRN), is one of the most common causes of FTD (Olszewska, et al., 2016). The goals of this research study focuses on understanding the molecular mechanisms underlying the etiology of FTD caused by mutations in *GRN*, therefore, we will focus on this subset of cases going forward.

Progranulin (PGRN) and its subunits

PGRN is a pleiotropic, secreted protein that has been implicated in wound healing and neuroprotection as well as anti-inflammatory pathways (Pogonowska et al., 2019). Structurally, PGRN is an ~88 kDa multi-functional glycoprotein composed of seven full-length granulin domains and one half-length paragranulin connected by peptide linker regions (Bateman and Bennett, 1998). Progranulin is composed of 7 granulin and 1 paragranulin subunits numbered GRN1 to GRN7. Previous studies have shown that progranulin can be cleaved by several proteases to generate individual granulins in the extracellular space (Zhu et al., 2002; Kessenbrock et al., 2008; Suh et al., 2012). Interestingly, these individual granulin subunits which make up the PGRN protein have been proposed to be functionally distinct from the precursor protein PGRN (Shoyab et al., 1990; Plowman et al., 1992; Culouscou et al., 1993).

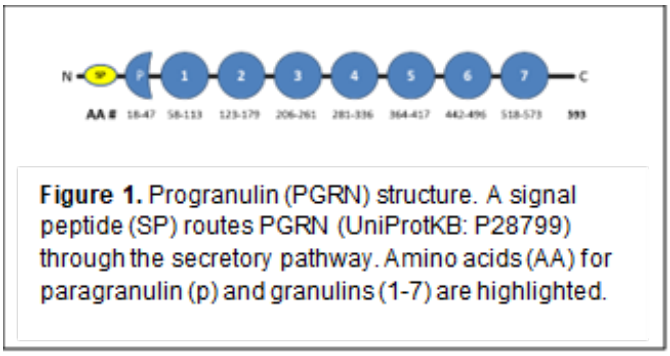


Figure 1. Progranulin (PGRN) structure. A signal peptide (SP) routes PGRN (UniProtKB: P28799) through the secretory pathway. Amino acids (AA) for paragranulin (p) and granulins (1-7) are highlighted.

Heterozygous mutations in the GRN gene cause FTD disease pathology through haploinsufficiency which leads to a ~50% reduction in PGRN mRNA, PGRN protein, and granulins in the body (Holler, et al., 2017). Various studies have found these pathogenic, loss-of-function GRN mutations in 5-20% of patients with a positive history of FTD and 1-5% of FTD patients with no family history of FTD (Baker et al., 2006; Rademakers et al., 2012). While there are heterogeneities within FTD pathology, heterozygous GRN mutation carriers always present with FTD-TDP (Mackenzie, et al.,

2011) meaning that these patients have pathological inclusions whose major component is TDP 43 (Goedert, et al., 2012).

TDP-43 is a ubiquitously expressed RNA-binding protein that is thought to be involved in gene regulation (Ou et al., 1995). It is predominantly found in the nucleus, though it normally shuttles between the nucleus and cytoplasm (Ederle et al., 2018). In patients with GRN mutation-FTD cases, TDP-43 is found mainly in the cytoplasm in a hyperphosphorylated, ubiquitinated, and truncated form (Hasegawa et al., 2008), and in addition to these TDP-43 neuronal cytoplasmic inclusions, there are short dystrophic neurites (Mackenzie et al., 2011) and general neuronal death. Although the presence of TDP-43 inclusions in the brains of patients with *GRN* mutations is well-characterized, it is currently unclear why the loss of progranulin function leads to an accumulation of TDP-43. Prior studies have shown that GRN mutations lead to changes primarily in the hippocampal, thalamic, and cortical regions of the brain by including the dysregulation of various proteins involved in inflammation, neuroprotection, and lysosomal function including GPNMB and Gal-3 (Huang, et al., 2020; Ward et al., 2017). The precise role of PGRN, and the pathways affected by the loss of function in FTD-GRN cases remains unclear. By further investigating the role of progranulin and the granulin subunits in a mouse model of FTD, the findings of this study will contribute to this gap in knowledge. Particularly novel, will be this study's inclusion of individual granulin subunits.

Granulin subunits

PGRN and GRNs work in this interesting balance. It is currently believed that PGRN has anti-inflammatory effects (Yoo et al., 2019), while the granulin subunits have proinflammatory effects (Pogonowska et al., 2019). There is active debate about

whether this statement holds true as recent data suggests GRNs may be required for cellular homeostasis (Holler et al., 2017). Even so, it is clear that the individual GRNs have specific roles and are independently functional. Prior studies have shown that a single point mutation in GRN2 can lead to axon growth dysfunction (Laird et al., 2010). Furthermore, granulin 3 has been implicated in having inhibitory or antagonistic effects of granulin 4 (Shoyab et al., 1990; Plowman et al., 1992; Culouscou et al., 1993). Granulin 4 has been shown to induce cell growth or inhibit cell proliferation depending on its cell lines (Shoyab et al., 1990; Plowman et al., 1992; Culouscou et al., 1992). In contrast, Granulins 5 and 7 have neurotrophic properties, and the complete deletion of granulin 7 from the full-length PGRN protein was shown to completely abolish the neurotrophic effect of PGRN (Wang et al., 2018). Granulin 6 has been implicated in regulating DNA synthesis in cultured astrocytes and glioblastoma cells (Liau et al., 2000). While it is generally understood that individual granulin subunits have a distinct function from each other, whether or not they play a role in FTD-GRN is still not well understood. Recent advances have shown that granulin subunits are depleted in human FTD-GRN patient brains (Holler et al., 2017) raising the possibility that replenishing these granulin subunits in progranulin deficient systems could rescue signs of dysfunction.

The Kukar lab was the first to report that full length PGRN is processed into its individual granulin domains in the lysosome (Holler, et al., 2017). These individual granulin subunits are more stable in the lysosome than the full length PGRN protein and are not degraded as quickly as the full-length protein (Holler, et al., 2017). This could imply that the granulin subunits are critical for lysosomal function and perhaps the

heterozygous mutations seen in FTD-GRN cases lead to decreased levels of these granulin subunits which then leads to lysosomal abnormalities. However, more research is necessary to investigate the specific role of progranulin and its granulins in FTD pathology. This thesis study will work to build upon the previous work in the lab to contribute to this goal.

Lysosomal Dysfunction in FTD

An important development in our understanding of FTD pathology, is the discovery that homozygous loss-of-function mutations in the GRN cause a lysosomal storage disease known as neuronal ceroid lipofuscinoses (NCLs) also known as Batten Disease (Mole and Haltia, 2015; Dolisca et al., 2013). NCL-GRN differs from FTD in that NCL has a juvenile onset meaning that the first symptoms can occur anytime between birth to young adulthood (Nita, et al., 2016) and NCL patients rarely live beyond their 40s (Cialone et al., 2012). The fact that complete loss of PGRN leads to lysosomal storage disorder, implies that PGRN and its granulin subunits are involved in lysosomal regulation. This suggests that FTD-linked heterozygous mutations in the *GRN* gene that lead to a reduction in the PGRN and granulin proteins might affect the lysosome and cause dysregulation of the lysosome leading to FTD-TDP.

Prior research has shown that FTD patients with *GRN* mutations show markers of lysosomal dysfunction in human FTD-GRN cases (Ward, et al., 2017). Post-mortem evidence of lysosomal dysfunction in the brain is an accumulation of lipofuscin (Moreno-Garcia, et al., 2018). Lipofuscins are age-pigments that generally collect in lysosomes following lysosomal dysfunction (Katz and Robison, 2002). Lipofuscin is extremely electron dense, and the composition of lipofuscin includes lipids, proteins, and metals,

but the precise components are not well understood. A consistently identified element of lipofuscin is subunit C or mitochondrial ATP synthase (SCMAS) (Elleder et al., 1997). As lipofuscins as a whole cannot be observed through a western blot, lipofuscin-related proteins can be measured as a proxy of lipofuscin accumulation (Bertolo, et al., 2019).

In addition to the widespread accumulation of lipofuscin, human FTD-GRN cases also show lysosomal enlargement (Ward, et al., 2017). Importantly, mouse models of FTD recapitulate several of these phenotypes of interest including lipofuscin accumulation and lysosomal enlargement. It has been shown that reliable markers of lysosomal dysfunction in Grn-KO mice are protein levels of Galectin-3 (Huang et al., 2020), and several different lysosomal enzymes including Cathepsin L (Holler, et al., 2017), Cathepsin Z (Huang et al., 2020), and Cathepsin D (Huang et al., 2020). In this study, we will be observing lysosomal enzyme levels and lysosomal dysfunction to characterize the hippocampus of 12-month old *Grn*-KO mice.

Markers of Lysosomal Dysfunction

Galectin-3 (Gal-3)

Galectins are a family of soluble carbohydrate-binding proteins (Aits et al., 2015). Galectin-3 (Gal-3) specifically differs from other galectins because structurally it contains one carbohydrate recognition domain and a non-lectin N-terminal peptide which induces Gal-3 self-association (Nio-Kobayashi et al., 2021). Gal-3 is a protein/complex sugar that can be used as a marker of lysosome dysfunction because it has been shown to translocate from the cytosol to the lysosome when the lysosome is damaged (Aits et al., 2015). Thus, higher levels of Gal-3 in the lysosome when compared to a control group indicates lysosomal dysfunction and damage.

Lysosomal associated membrane protein 1 (LAMP-1)

LAMP-1 is a highly glycosylated protein found in the lysosomal membrane (Meikle et al., 1997). The lysosomal membrane is critical as it is responsible for maintaining the acidic environment within the lysosome, sequestering the active lysosomal enzymes, and regulating the transport of lysosomal products to the cytoplasm (Andrejewski et al., 1999). Within the membrane, LAMP1 is known to have a highly glycosylated lumenal domain and a short cytoplasmic domain (Cook et al., 2004). Functionally, LAMP-1 is thought to help maintain the integrity of the lysosomal membrane (Meikle et al., 1997). Thus, lower levels of LAMP-1 in the lysosome when compared to a control group are associated with lysosomal dysfunction as it indicates that the amount or size of lysosomes decreases and lysosomal biogenesis is disrupted (. Fluctuations in LAMP1 have been implicated in lysosomal dysfunction with decreases hypothesized to indicate x, y, and z, and increases hypothesized to indicate lysosomal enlargement.

Subunit c of mitochondrial ATP synthase (SCMAS)

SCMAS is a hydrophobic protein that is involved in oxidative phosphorylation in the mitochondria (Ryazantsev et al., 2007). In lysosomal storage disorders such as infantile and juvenile neuronal ceroid lipofuscinosis (NCL), an abnormal accumulation of SCMAS has been found in the lysosome (Ryazantsev et al., 2007). Thus, higher levels of SCMAS in the lysosome when compared to a control group indicates lysosomal dysfunction.

Cathepsin D (CAT D)

Cathepsin D is a soluble lysosomal aspartic endopeptidase that is active within the acidic compartment of the lysosome (Benes et al., 2008). The mature active lysosomal protease is composed of heavy (34 kDa) and light (14 kDa) chains that are linked by non-covalent interactions (Erickson et al., 1981; Conner et al., 1992; Grieselmann et al., 1985). Under normal physiological conditions, CatD is thought to assist in protein degradation within the lysosome which is a critical function of the lysosome (Benes et al., 2008). CatD deficiency or lower levels of CatD in the lysosome when compared to a control group indicates lysosomal dysfunction.

Cathepsin B (CAT B)

Cathepsin B is a cysteine based lysosomal protease that is known to inhibit the expression of autophagy-related proteins (Cermak et al., 2016). By regulating the degradation of unnecessary or dysfunctional cellular components (Yadati et al., 2020; Man et al., 2016), CatB assists in lysosomal biogenesis and is important for the lysosome to maintain its structural and functional elements (Man et al., 2016; Karageorgos et al., 1997). Thus, lower levels of CatB in the lysosome when compared to a control group indicates lysosomal dysfunction.

Cathepsin Z (CAT Z)

Cathepsin Z is a cysteine protease found within the lysosome (Bhutani et al., 2012; Brix et al., 2005). It is specifically a carboxypeptidase that assists in the rapid degradation of proteins (Brix et al., 2005). Lower levels of CatZ in the lysosome when compared to a control group is an indicator of lysosomal dysfunction.

Gliosis and Inflammation in FTD

Neuroinflammation and gliosis are other features seen in patients with NCL-GRN and FTD-*GRN* (Groh et al., 2013; Groh et al., 2016). In fact, one method of measuring the effectiveness of potential treatments of NCL in mice models is measuring the reduction of neuroinflammation and gliosis pre- and post- treatment (Seehafer et al., 2011). Similarly, in cases of FTD-GRN, human imaging studies have shown that neuroinflammation begins early in disease pathogenesis while gliosis occurs specifically during end-stage pathology (Cagnin et al., 2004). The exact mechanism behind this neuroinflammation and gliosis is still unknown, however, it is a goal of many therapeutic interventions to reduce or address levels of inflammatory markers.

Markers of Gliosis and Inflammation

Ionized calcium-binding adapter molecule 1 (IBA1)

Microglia play a critical function in the brain because in injured or disease states, microglia will transform from a resting to an active state in order to release cytokines and become phagocytic (Kreutzberg et al., 1996; Raivich et al., 1999). Ionized calcium-binding adapter molecule 1 (IBA1) is a calcium-binding protein that is expressed in the microglia (Imai et al., 1996; Kohler et al., 2007) and participates in phagocytosis in activated microglia (Ohsawa et al., 2004). Studies have shown that IBA1 protein levels increase in activated microglia/macrophage, making IBA1 a reliable marker of gliosis and inflammation (Ito et al., 2001). Prior studies have shown that the degree of microglial pathology as indicated by IBA1 correlates with the rate of disease progression (Brettschneider et al., 2012). Thus, higher levels of IBA1 in brain tissue when compared to a control group is an indicator of gliosis and inflammation.

Glial fibrillary acidic protein (GFAP)

Astrocytes are a type of glial cell found in the central nervous system (Yang et al., 2015). Glial fibrillary acidic protein (GFAP) is an intermediate filament protein found in astrocytes (Tzeng et al., 2005), and GFAP gene activation plays a critical role in the astroglia cell activation that occurs after neurodegeneration (Yang et al., 2015), and activated astrocytes express high levels of GFAP (Tzeng et al., 2005). Excessive gliosis occurs during a neuroinflammatory response (Sofroniew et al., 2009). Thus, higher levels of the GFAP protein is a reliable marker of gliosis when compared to a control group.

Cluster of differentiation 68 (CD68)

Macrophages are key components of the innate immune system and play a critical role in inflammation (Fujiwara and Kobayashi, 2005). Cluster of differentiation 68 (CD68) is a protein that is highly expressed in macrophages (Chistiakov et al., 2017). CD68 is a heavily glycosylated protein (Holness et al., 1993) that has been proven to be a reliable marker of inflammation (Fujiwara and Kobayashi, 2005). Higher levels of CD68 in brain tissue when compared to a control group is an indicator of inflammation.

Pre-clinical Mice Model of FTD

Complete PGRN deficiency in mice has proven to be a reliable preclinical mouse model of FTD, and it has been well characterized (Ding, et al., 2010). These Grn-KO mice share some of the fundamental characteristics of human patients with FTD including signs of lysosomal dysfunction, lipofuscinosis, microgliosis, and neuroinflammation (Arrant, et al., 2018). Assessment of protein levels via immunoblot of whole brain lysate from GRN-WT and GRN-KO mice has shown that there are

dysregulations of several proteins also identified in human cases including Cathepsin D, LAMP1, Galectin 3, GFAP, and CD68 (Huang et al., 2020; Gotzl et al., 2014). `

AAV-Grn Injections Rescue Function

Interestingly, several studies have shown that adeno-associated virus-mediated gene replacement of the full length granulin gene in adult mice (AAV-GRN) rescues some lysosomal dysfunction seen in Grn-KO mice (Arrant et al., 2018; Logan et al., 2021). Similarly, the administration of full length PGRN proteins that were engineered to cross the blood brain barrier can rescue some lysosomal function in Grn-KO mice (Logan, et al., 2021). This suggests the potential for treatments of FTD which can target not just patient symptoms, but the underlying cause of the disorder.

While previous studies have shown that the replacement of full length progranulin can rescue lysosome dysfunction (Arrant, et al., 2018), it is unclear whether the replacement of a single granulin domain subunit would be beneficial. The goal of this study is to determine if AAV injection of the granulin-2 subunit (AAV-GRN-2) in neonatal *Grn*-KO mice is able to similarly rescue lysosomal function and alleviate the deleterious effects of loss of progranulin which can be measured by comparing lipofuscin aggregates as well as known markers of lysosomal dysfunction.

Taking into consideration the work conducted in our lab and others, I hypothesize that the addition of granulin subunits (GRN2 and GRN4) will rescue known signs of lysosomal dysfunction and gliosis in *Grn*-KO mice. If my hypothesis is proven correct, I expect that markers increased in FTD deficiency like Galectin-3 protein levels will be decreased compared to knockout animals treated with GFP control. Addictionally, I expect that markers of gliosis seen to be increased when PGRN is lost like GFAP will

be decreased when both PGRN and granulin subunits are expressed. The conclusions of this work will help us to understand the role that granulins play in the brain, and it may open doors to future studies for novel therapeutics for treating FTD.

Methods

AAV-injection of Mice[1]

$Grn^{-/-}$ used in this study were originally generated in Dr. Aihao Ding's laboratory and purchased from the Jackson Laboratory. Mice were housed in the Department of Animal Resources at Emory University and all work was approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

We generated 4 AAV constructs for this study. These constructs were control (GFP alone), PGRN (GFP+PGRN), GRN2+L# (GFP + GRN2 linker 3) and GRN4+L5 (GFP + GRN4 linker 5). P0 mouse pups were injected with 1 microliter AAV construct of interest either GFP, PGRN, GRN2+L3 or GRN4+L5 bilaterally into the lateral ventricle using a free hand injection. Mice were sacrificed after 12 months and brains were immediately dissected from the skull and frozen at -80C. Whole brains were later thawed on ice and cortical, hippocampal, and thalamic sections were bulk dissected from the skull and frozen immediately at -80C. Frozen hippocampal brain samples from 12-month old $Grn^{+/+}$ wild type (n=27) and $Grn^{-/-}$ knock out (n=44) mice were allocated for further processing.

Mouse Brain Sample Processing

To prepare samples for the immunoblot analysis of proteins, a novel protocol was

[1] All mice injections were performed by PhD candidate Jessica Root.

developed in which approximately 40 mg of mouse hippocampal tissue from each sample was placed in a solution of PBS with added HALT phosphatase protease inhibitor (PPI) at a dilution of 1:2 (weight to volume). The PPI was diluted into the 1xPBS at 1:100. In the PBS + PPI solution, the sample was cut into smaller pieces with mini scissors. Once cut into smaller pieces, the sample is ready for further homogenization.

A bead lysis kit was used for the homogenization of these small, soft hippocampal samples. The samples were cut into pieces and pipetted into 1.5 mL RINO screw-tap tubes (Next Advance) prefilled with zirconium oxide beads. Tubes were placed into the Bullet Blender (Next Advance) for homogenization.

Once homogenized, the recovered homogenate was diluted 1:5 in the RIPA lysis buffer supplemented with 1x HALT protease and phosphatase inhibitor. After 15 minutes, the solution was sonicated at 30A for 2 seconds on and 8 seconds of rest with a total of 10 seconds. After sonication, the solution was spun down in a centrifuge at 20,000 RCF at 4C for 10 minutes. Protein concentration was measured with the bicinchoninic acid (BCA) assay, and 71 samples were frozen in aliquots at -80C.

Immunoblot

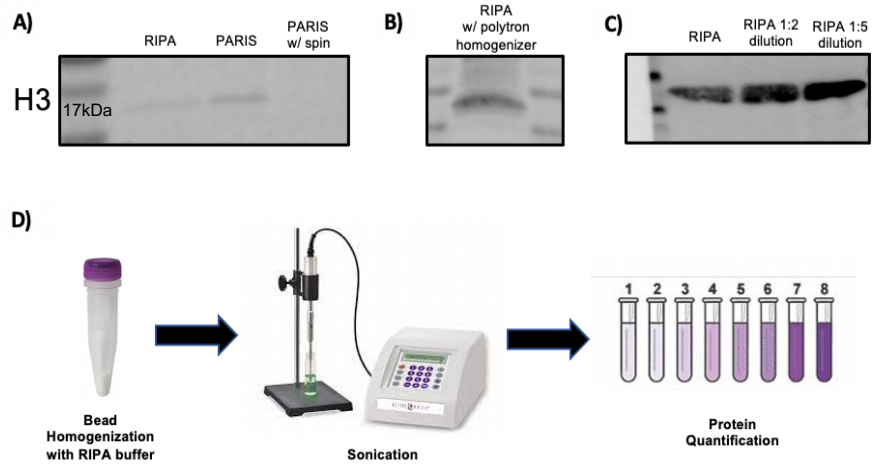
Samples were prepared for immunoblot in 1X Laemmli loading buffer with 20 mM tris(2-carboxyethyl)phosphine (TCEP)) followed by denaturation at 70 degrees C for 15 minutes. Protein samples were separated on Bio-Rad 4-20% 26-well gels at 100 V and transferred to a 0.2 micron nitrocellulose membrane using the Bio-Rad Trans-blot Turbo system. Membranes were stained with either total protein stain or H3 (company, catalog #, working concentration (microg/mL)) antibodies and imaged on the Odyssey

Fc (Licor) to assess transfer efficiency and normalize protein abundance between samples. After blocking with BulletBlock (Nalacai) for 30 minutes at room temperature, membranes were incubated overnight at 4C with primary antibodies (Supplemental Table 1). All primary antibodies were diluted in 1:1 glycerol. Either a total protein stain or H3 was used as loading controls. Near-infrared fluorescent secondary antibodies (diluted in TBST) or HRP-conjugated (diluted in 0.5% milk in TBST) antibodies were incubated for 1 hour at room temperature: (Supplemental Table 1). For HRP visualization, blots were incubated in WesternSure PREMIUM Chemiluminescent Substrate (926-95010, Licor) for 5 min before imaging. Near-infrared or chemiluminescent blots were imaged using Odyssey Fc (Licor) and analyzed by Image Studio software 5.2.

RESULTS

Bead homogenization with RINO tubes is an effective lysis strategy for hippocampal tissue. A novel protocol was developed to prepare hippocampal sections for immunoblotting. We aimed to produce a whole cell lysate. The efficiency of the lysis strategy was determined by evaluating the amount of Histone 3 (H3) which is a marker of the nuclear compartment,

Figure 1: Development of hippocampal section lysis strategy
Histone 3 (H3) immunoblots utilizing different lysis techniques: PARIS Kit (A) polytron homogenizer (B) and bead homogenization (C). Final lysis strategy used for all hippocampal sections (D)



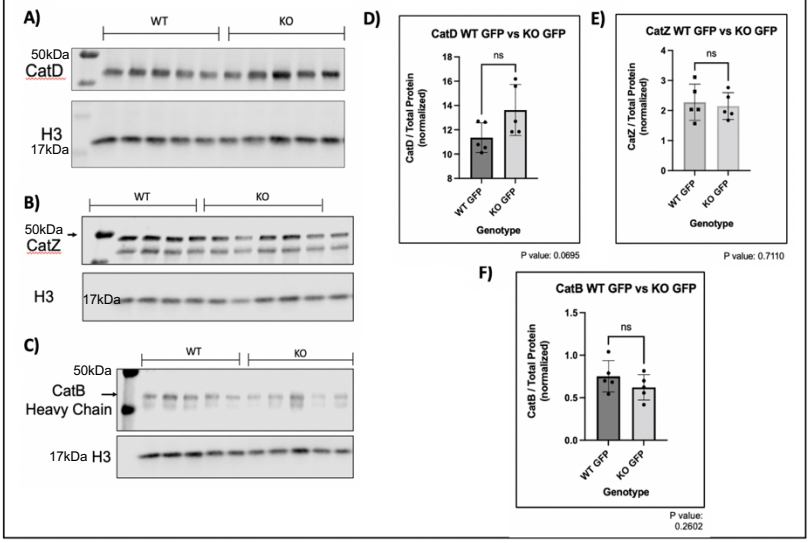
present when equal amounts of protein from varying lysis conditions was loaded. The PARIS kit was thought to effectively extract protein from soft tissue; however, both the PARIS kit and the polytron homogenizer were unable to produce immunoblots without low levels of H3 or significant background which interfered with signal detection of hippocampal tissue (Figure 1A; Figure 1B). It was found that the highest levels of H3 with the least amount of background were detected in the hippocampal tissue when bead homogenization was used in combination with RIPA lysis buffer diluted by a factor of 1:5 (Figure 1C).

Lysosomal enzyme levels were unchanged in the hippocampus between WT and *Grn* KO mice at 12 months.

Cathepsin D, Cathepsin Z, and Cathepsin B are enzymes that are critical for lysosomal function. In prior studies, their levels were found to be significantly altered in whole brain lysates of *Grn* KO mice at 12 months of age (Huang et al, 2020). As such, rescue of cathepsin levels may indicate rescue of lysosome function for our samples. We first characterized whether there were differences between the various cathepsins in the WT and KO control groups at baseline. Quantification

Figure 2: Characterization of lysosomal enzyme levels in the hippocampal tissue of *Grn* KO mice at 12 months

Immunoblots indicating levels of lysosomal enzymes in WT and KO genotypes for Cathepsin D (A), Cathepsin Z (B), Cathepsin B (C). Bar graphs indicating levels of lysosomal enzymes in WT and KO genotypes for Cathepsin D (D), Cathepsin Z (E), Cathepsin B (F)



from immunoblots targeting these cathepsins were evaluated using an unpaired t-test comparing the WT-GRP and KO-GFP treatment groups. I found that levels of Cathepsin D, Cathepsin Z, and Cathepsin B were not significantly different between WT and *Grn* KO mice in the hippocampus at 12 months (Figure 2A-F). These are lysosomal enzymes that if upregulated would indicate lysosomal dysfunction implying that at 12 months of age, lysosomal enzymes are not dysregulated in hippocampal tissue of *Grn* KO mice. Though these enzyme levels were not found to be significantly altered between the WT and KO genotype, this did raise the question of whether or not the addition of granulin subunits led to lysosomal dysregulation by increasing levels of these enzymes in hippocampal tissue.

AAV-GRN subunits did not increase lysosomal enzyme levels in FTD-like mice.

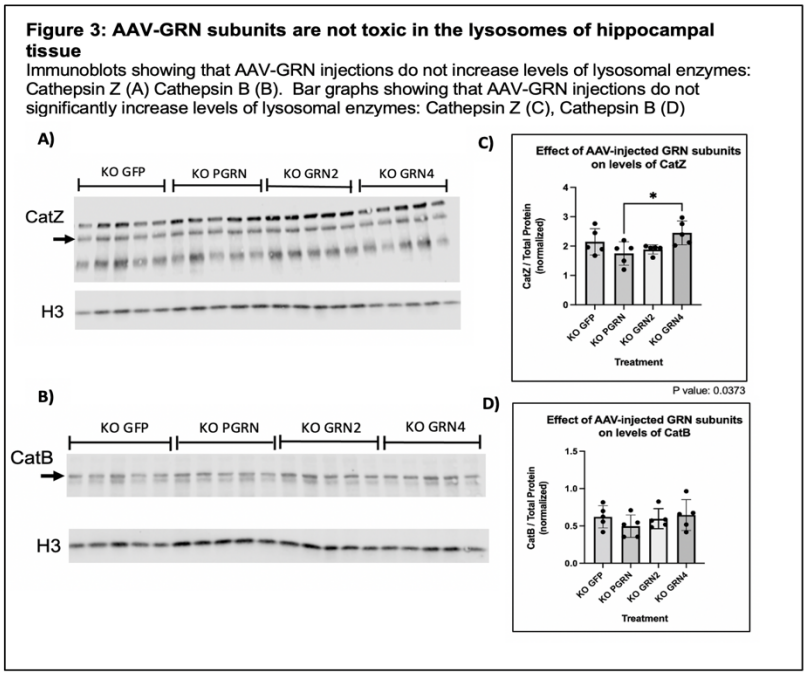


Figure 3: AAV-GRN subunits are not toxic in the lysosomes of hippocampal tissue
Immunoblots showing that AAV-GRN injections do not increase levels of lysosomal enzymes: Cathepsin Z (A) Cathepsin B (B). Bar graphs showing that AAV-GRN injections do not significantly increase levels of lysosomal enzymes: Cathepsin Z (C), Cathepsin B (D)

Previous studies have hypothesized that the overexpression of granulin subunits may be detrimental. Therefore, it is critical to determine whether the expression of granulin subunits using AAVs leads to exacerbation of known disease-like phenotypes in Grn-KO mice. Importantly, even markers like cathepsins which were not found to be significantly different in WT and KO control mice could be affected when individual

granulins are expressed. To evaluate this possibility, a one-way ANOVA was performed comparing Grn-KO mice injected with GFP, PGRN, GRN2, and GRN4. The addition of GRN2 and GRN4 subunits did not significantly increase the levels of lysosomal enzymes Cathepsin Z and Cathepsin D in the hippocampus of Grn KO mice (Figure 3A-D). This provides preliminary evidence that GRN subunits may not be toxic within the lysosomes as previously thought (Yoo et al., 2019). If GRN2 or GRN4 were toxic, we would expect that lysosomal dysfunction and therefore lysosomal enzyme activity would increase with the addition of granulin subunits, which we do not see within the hippocampus of *Grn* KO mice at 12 months (Figure 3A-D). Interestingly, GRN4 does significantly increase levels of Cathepsin Z when compared to the KO PGRN treatment (Figure 3C) in hippocampal tissue; however, GRN4 does not significantly increase levels of Cathepsin Z when compared to the FTD-like mice with the KO GFP treatment supporting the idea that GRN4 does not exacerbate lysosomal dysfunction in the hippocampus in FTD-like mice at 12 months.

Lysosomal membrane protein levels were significantly altered in the hippocampus between WT and *Grn* KO mice at 12 months

Lysosomal enzymes are just one metric that can be used to evaluate lysosomal function in models of FTD. Another sign of dysfunction observed across models of FTD caused by granulin mutations includes the dysregulation of proteins found in the lysosomal membrane. Galectin-3 and LAMP1 are both lysosomal membrane proteins whose levels were found to be significantly different in the hippocampi of WT and *Grn* KO mice at 12 months (Figure 4A-D). Galectin-3 was significantly upregulated in the FTD-like mice indicating that the *Grn* KO mice had higher levels of lysosomal dysfunction in the hippocampal tissue (Figure 4A; Figure 4C). LAMP1 was significantly downregulated in FTD-like mice indicating that *Grn* KO mice had higher levels of lysosomal dysfunction in the hippocampal tissue (Figure 4B; Figure 4D). This finding conflicts with previous reports which found that LAMP1 was increased in models of FTD caused by granulin deficiency, and observations in human cases which display increased lysosomal size

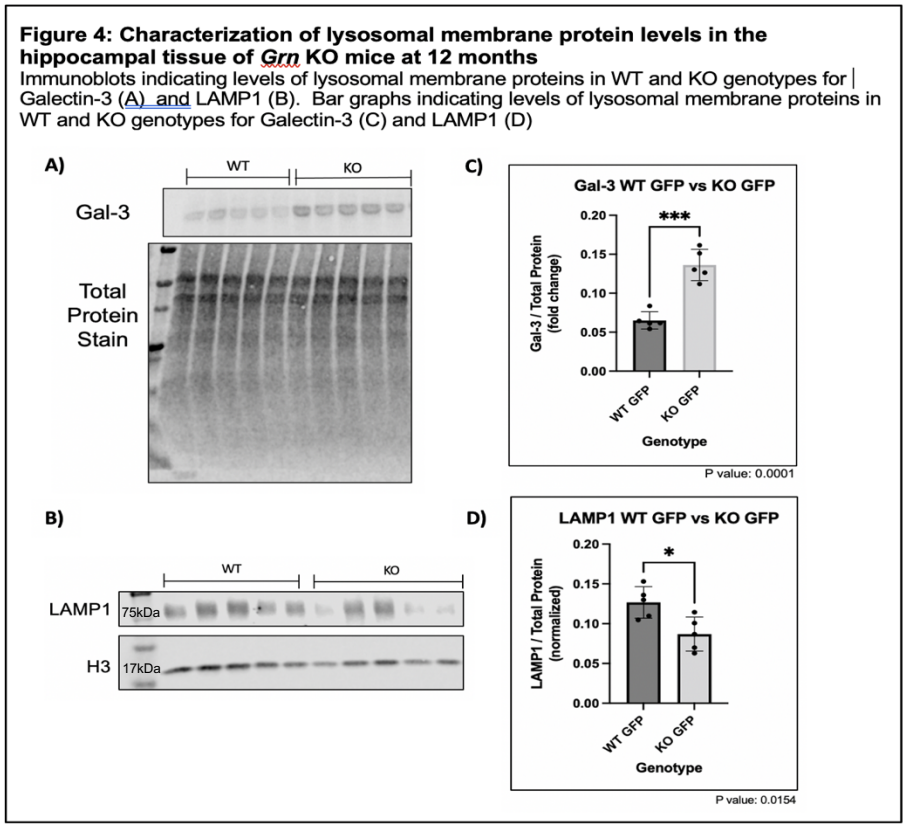


Figure 4: Characterization of lysosomal membrane protein levels in the hippocampal tissue of *Grn* KO mice at 12 months
Immunoblots indicating levels of lysosomal membrane proteins in WT and KO genotypes for Galectin-3 (A) and LAMP1 (B). Bar graphs indicating levels of lysosomal membrane proteins in WT and KO genotypes for Galectin-3 (C) and LAMP1 (D)

and number. However, LAMP1 levels may still be a valid marker to evaluate the ability of GRNs to rescue lysosomal function in *Grn*-KO mice.

Granulin subunits can rescue lysosomal dysfunction

The Grn-KO mice in this study were found to have significantly upregulated levels of Gal-3 (Figure 5B) which is a reliable marker of lysosomal dysfunction. Once reliable markers were identified, the critical question of this study could be evaluated. This study is the first to question whether the expression or individual granulin subunits are able to ameliorate signs of lysosomal dysfunction *in vivo.* Using a one-way ANOVA comparing the levels of all injection treatment groups (WT-GFP, KO-GFP, KO-GRN2, and KO-GRN4), it was determined that the AAV-GRN2 injections were able to rescue Gal-3 back to levels seen in WT GFP (p value < 0.0021) (Figure 5A-B). Similarly, the AAV-GRN4 injections were also able to rescue Gal-3 protein levels in the hippocampal tissue of *Grn* KO mice back to WT levels but with higher significance (p value < 0.0005) (Figure 5A-B). There was one data point from the KO PGRN injected group that was excluded as it was found to be an outlier through statistical analysis. The data with the outlier included can be found in Supplemental Figure 1. This is the first evidence to suggest that an individual granulin subunit may be sufficient to rescue lysosomal dysfunction in a mouse model of FTD. I next moved to

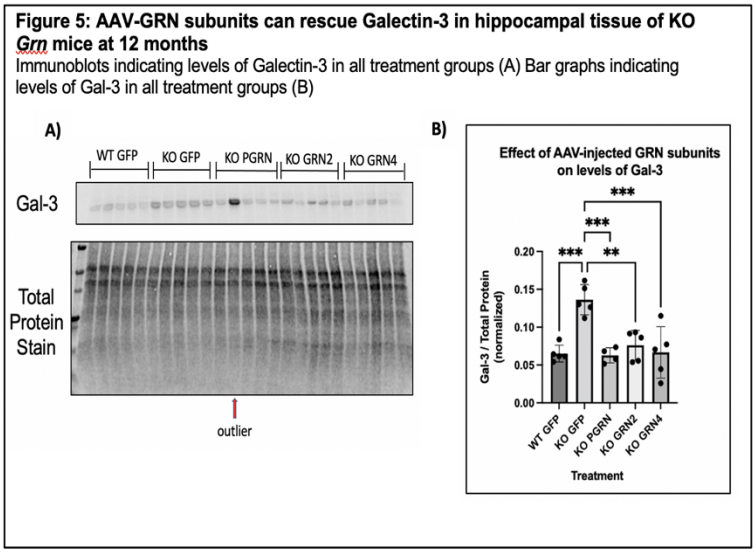


Figure 5: AAV-GRN subunits can rescue Galectin-3 in hippocampal tissue of KO *Grn* mice at 12 months
Immunoblots indicating levels of Galectin-3 in all treatment groups (A) Bar graphs indicating levels of Gal-3 in all treatment groups (B)

evaluate whether granulin subunits had a similar effect on additional targets that were confirmed to be differentially expressed in WT and KO mice injected with GFP.

Granulin subunits do not rescue LAMP1 levels in *Grn* KO mice at 12 months.

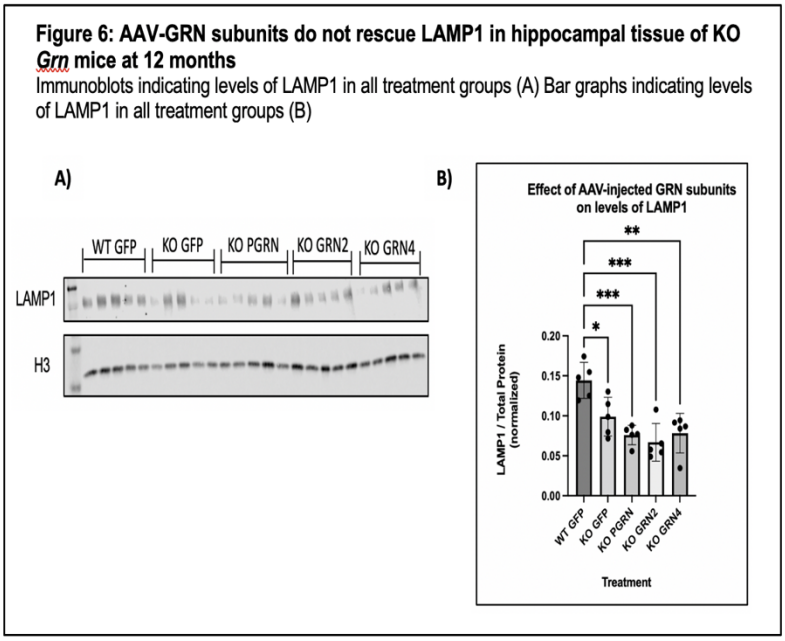


Figure 6: AAV-GRN subunits do not rescue LAMP1 in hippocampal tissue of KO *Grn* mice at 12 months
Immunoblots indicating levels of LAMP1 in all treatment groups (A) Bar graphs indicating levels of LAMP1 in all treatment groups (B)

Grn KO mice were found to have significantly reduced levels of LAMP1 protein in hippocampal tissue at 12 months when compared to the KO mice injected with GFPWT mice (Figure 6A-B). Decreased levels of LAMP1 is a reliable marker of lysosomal dysfunction. The addition of AAV-GRN2 and AAV-GRN4 did not significantly alter levels of LAMP1 when compared to the KO mice injected with GFP (Figure 6A-B). The mice that received PGRN, GRN2, and GRN4 had LAMP1 levels that remained significantly lower than the WT-GFP mice. This data shows that the addition of these granulin subunits does not ameliorate nor exacerbate the lysosomal dysfunction seen in FTD-like mice by altering levels of LAMP1 in hippocampal tissue at 12 months of age (Figure 6A-B).

The addition of GRN subunits to *Grn* KO mice at 12 months decreases levels of Cathepsin D

Increased levels of Cathepsin D heavy chain are a reliable marker of lysosomal dysfunction across models of progranulin deficiency. While FTD-like mice did not have significantly increased levels of Cathepsin D when compared to the WT group (p value < 0.0695) (Figure 2D), there was an intriguing trend of increased protein levels. When a one-way ANOVA was performed to ask whether there was any significant effect of granulin expression, I found that the addition of AAV-GRN2 and AAV-GRN4 did significantly decrease levels of Cathepsin D when compared to the FTD-like mice (Figure 7B). Interestingly, these granulin subunits do not decrease levels of Cathepsin D significantly below the levels found in the WT group (Figure 7A-B). Taken together with the trending increase in Cathepsin D levels observed between WT-GFP and KO-GFP mice, this data provides strong preliminary evidence that granulin subunits can ameliorate levels of Cathepsin D in mouse models of FTD.

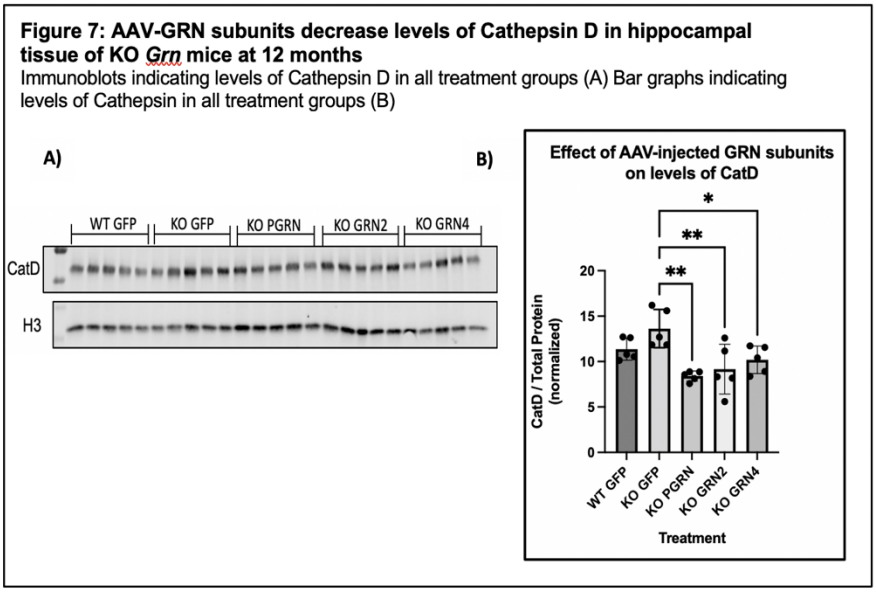


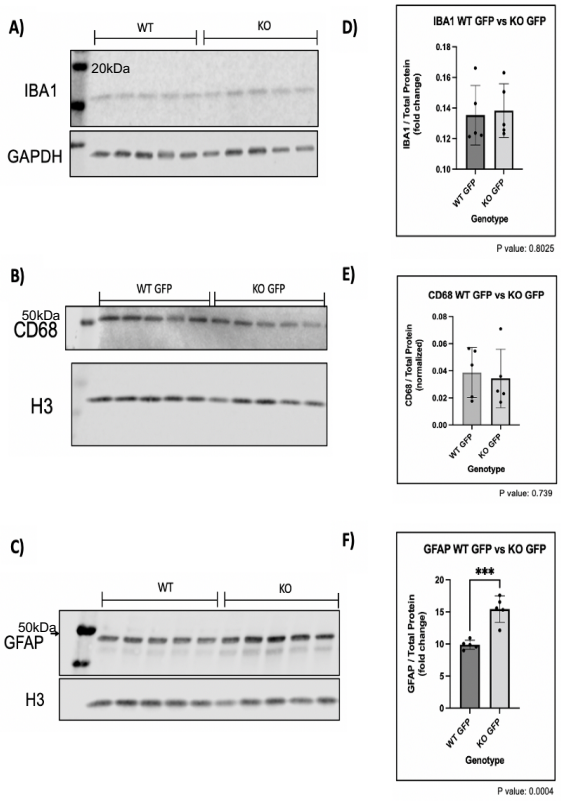
Figure 7: AAV-GRN subunits decrease levels of Cathepsin D in hippocampal tissue of KO *Grn* mice at 12 months
Immunoblots indicating levels of Cathepsin D in all treatment groups (A) Bar graphs indicating levels of Cathepsin in all treatment groups (B)

Astrocytosis significantly increased in hippocampal tissue of *Grn KO* mice, but microliga did not show a similar increase after 12 months

Increased levels of GFAP is a reliable marker of activated astrocytes. In *Grn* KO mice, there were higher levels of GFAP protein when compared to the WT mice suggesting that GFAP may serve as a reliable phenotype of FTD in *Grn* KO mice (Figure 8C and 8F). Interestingly, while astrocytes seem have increased activation in the hippocampal tissue, markers of microglia seem to be unaffected. The levels of IBA1 and CD68, both reliable indicators of microglia and one marker of microgliosis and inflammation, were unchanged between the two genotypes (Figure 8A-E).

Figure 8: Characterization of inflammation and gliosis in the hippocampal tissue of *Grn* KO mice at 12 months

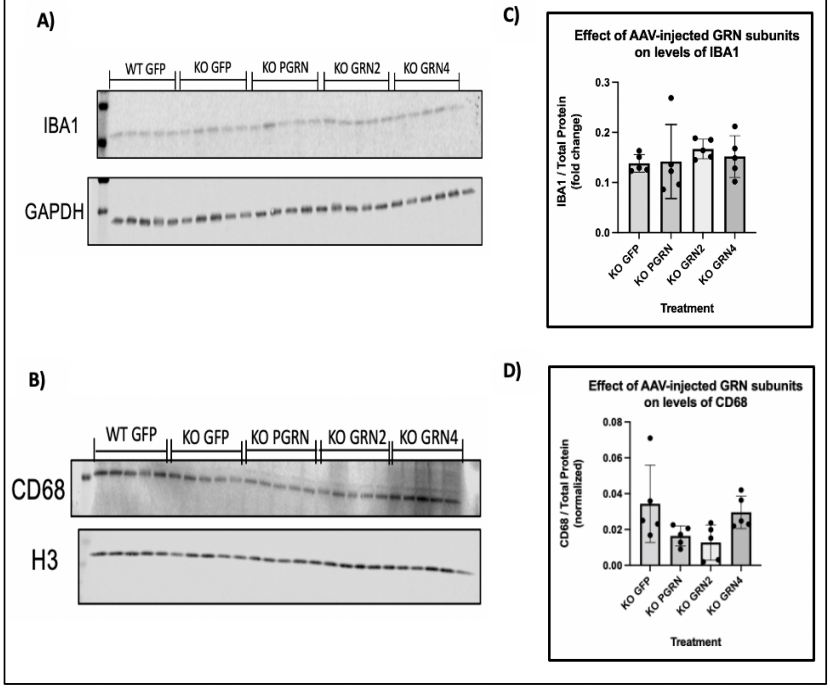
Immunoblots indicating protein levels in WT and KO genotypes for IBA1 (A), CD68 (B), GFAP (C). Bar graphs indicating protein levels in WT and KO genotypes for IBA1 (D), CD68 (E), GFAP (F)



AAV-GRN subunits did not exacerbate microgliosis or inflammation in FTD-like mice.

AAV-GRN injections did not significantly alter levels of IBA1 or CD68 in hippocampal tissue of *Grn* KO mice at 12-months old (Figure 9A-D). This suggests that the addition of granulin subunits does not increase microgliosis or inflammation (Figure 9A-D).

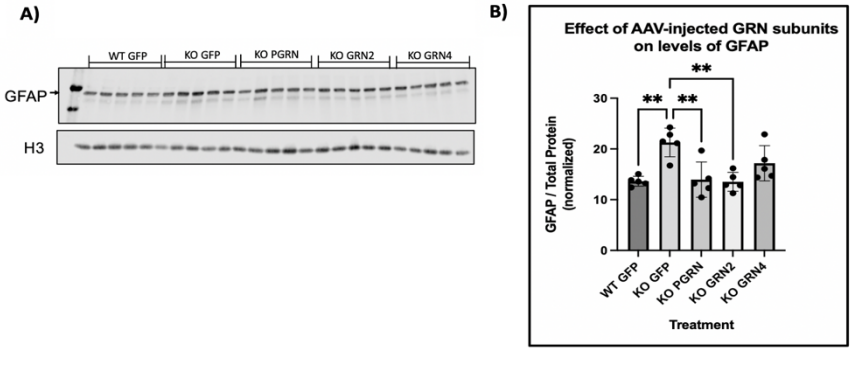
Figure 9: AAV-GRN subunits do not exacerbate inflammation and gliosis in the hippocampal tissue of *Grn* KO mice at 12 months
Immunoblots showing that AAV-GRN injections do not increase protein levels marking inflammation and gliosis in WT and KO genotypes: IBA1 (A) and CD68 (B). Bar graphs indicating that AAV-GRN injections do not significantly increase protein levels that mark inflammation and gliosis in WT and KO genotypes: IBA1 (C) and CD68 (D).



AAV-GRN2 can rescue markers of astrogliosis in FTD-like mice

This study found that there were significantly higher levels of GFAP, a reliable indicator of activated astrocytes, in *Grn* KO mice. The addition of AAV-GRN2 injections were able to significantly reduce levels of GFAP back to what was seen in WT mice (Figure 10A-B). AAV-GRN2 injections can rescue astrogliosis.

Figure 10: AAV-GRN subunits can rescue GFAP in hippocampal tissue of *Grn* KO mice at 12 months
Immunoblots indicating levels of GFAP in all treatment groups (A) Bar graphs indicating levels of GFAP in all treatment groups (B)



Interestingly, AAV-GRN4 injections did not significantly alter levels of GFAP (Figure 10A-B) supporting the idea that individual granulin subunits have distinct functions from each other.

DISCUSSION

In frontotemporal dementia, degeneration of grey matter is generally concentrated in the frontal and temporal lobes, affecting the hippocampal structures. The hippocampus is a complex structure that is found within the temporal lobe of the brain. It has long been studied and understood to be critical for learning and memory (Fumagalli et al., 2012; Anand and Dhikav, 2012). The hippocampus is of particular interest in diseases and disorders where memory function is affected. Previous studies have shown that late stage preclinical mouse models of FTD display some deficits in learning and memory (Wils, 2012). Additionally, 12 month old *Grn*-KO mice have been shown to have increased inflammatory markers, as well as increased lipofuscinosis in the CA3 region of the hippocampus, which was ameliorated with the administration of full length progranulin (Arrant, 2018). Though these findings suggest that the hippocampus is dysfunctional in 12-month *Grn*-KO mice and that replacing progranulin *in vivo* could address this dysfunction, many questions remain which are addressed in this study. First, in this study, the extent of lysosomal dysfunction in the hippocampus has been expanded upon to include several lysosomal enzymes and markers of lysosomal membrane integrity. Second, this study examines markers of inflammation and gliosis to understand how the hippocampus specifically is altered in 12-month *Grn*-KO mice. Third, this study is the first to ask whether an individual granulin subunit can ameliorate the dysfunction characteristic in *Grn*-KO mice.

Previous studies by our lab have shown that Galectin-3 is a reliable marker of lysosomal dysfunction in *Grn*-KO mice brains, which have shown detectable increases in protein levels starting at 6 months of age (Huang et al., 2020). These analyses relied on ELISAs to quantify protein levels and used whole brain lysates. This study was able to demonstrate that Galectin-3 is significantly upregulated between the WT and KO genotypes transduced with GFP encoded AAV, where the FTD-like *Grn*-KO mice express more Galectin-3 specifically in the hippocampal tissue. Additionally, this quantification was done using immunoblot, confirming an additional modality through which this phenotype can be observed in 12-month old mice.

An innovative and novel aspect of this study design was the addition of individual granulin subunit treatment groups. While previous studies have shown that administering progranulin to knockout mice is able to ameliorate levels of lipofuscin, a marker of lysosomal dysfunction, in the hippocampus, the ability of a granulin subunit to have similar benefits on markers of lysosomal dysfunction was unknown. We found that the AAV-GRN2 injections as well as the AAV-GRN4 injections were able to rescue Galectin-3 to levels that were observed in the WT mice. This is the first evidence that granulin subunits are able to rescue lysosomal dysfunction in a preclinical model of FTD.

Prior studies have also shown that GFAP is a reliable indicator of astrogliosis. In this study, 12-months-old *Grn* KO mice were found to have significantly higher levels of GFAP than the WT genotype suggesting that astrogliosis is a reliable phenotype of FTD in preclinical mouse models. Furthermore, we showed that AAV-GRN2 injections were able to significantly decrease GFAP levels in *Grn*-KO mice back to levels observed in

WT mice. This data further suppors the idea that granulin subunits can serve a therapeutic function. Interestingly, AAV-GRN4 did not decrease levels of activated astrocytes. Taken together with previous work, this is another piece of evidence to suggest that granulin subunits are functionally distinct from each other. While *Grn* KO mice did have higher levels of astrogliosis, there were no significant differences found in levels of IBA1 and CD68 between the WT and KO mice. These are reliable markers of microgliosis. This data raises the possibility that within the hippocampus of 12-month-old *Grn* KO mice, microgliosis is not a reliable phenotype of FTD.

Several markers of lysosomal function which were unchanged in the hippocampus between the WT and KO genotypes of FTD-like mice include Cathepsin B and Cathepsin Z. Prior studies indicated that these lysosomal enzymes were significantly altered in whole brain lysates of *Grn*-KO mice at 18 months of age (Huang et al, 2020). The data in this study raises the possibility that PGRN haploinsufficiency does not lead to changes in Cathepsin B and Cathepsin Z within the hippocampus at least within the timeframe used in this study; however, a key difference between this work and our previous findings is the age of the mice. It is possible that cathepsins B and Z could be dysregulated in the hippocampus at 18 months of age when lysosomal dysfunction is generally more pronounced. Furthermore, this data does not exclude the possibility that levels could be dysregulated in other areas of the brain such as the prefrontal cortex or the thalamus. Importantly, Cathepsin D, another marker of lysosomal dysfunction, while not significantly altered between the GRN-KO and WT genotypes, did indicate a trending level of upregulation which might have become significant had the FTD-like mice been allowed to mature beyond the 12 months used in

this study. We found that the AAV-GRN2 injections as well as the AAV-GRN4 injections were able to significantly decrease Cathepsin D in *Grn*-KO mice to levels that were observed in the WT mice. Ultimately, we were able to show that lysosomal dysfunction as indicated by altered levels of Cathepsin B or Cathepsin Z within the hippocampus may not be a reliable phenotype of FTD in 12-month-old *Grn*-KO mice, and granulin subunits 2 and 4 decreases Cathepsin D levels in the hippocampus of 12-month-old *Grn*-KO mice raising the possibility that Cathepsin D could be a reliable phenotype of FTD in *Grn*-KO mice at 18 months.

As there was no significant difference between Cathepsin B and Cathepsin Z levels between GRN-KO and WT genotypes, we could not determine if the addition of granulin subunits was able to rescue these protein levels; however, we were able to compare the AAV-GRN2 and AAV-GRN4 injected mice to the KO mice to better understand whether or not granulin subunits had a harmful effect. Several prior studies had suggested that granulin subunits were pro-inflammatory or potentially toxic (Yoo et al., 2019; Kao et al., 2017); however, in this study we found that the addition of granulin subunits did not exacerbate levels of lysosomal enzymes beyond what was seen in the KO-GFP mice nor did the addition of granulin subunits increase inflammation or gliosis within the hippocampal tissue of *Grn*-KO mice at 12 months. This raises the possibility that the administration or expression of granulin subunits are not harmful or toxic.

While this study does inform our understanding of lysosomal dysfunction within the hippocampus of FTD-like mice and the potential for granulin subunits to ameliorate or rescue function, it is still unclear as to whether granulin subunits can rescue function in areas of the brain outside of the hippocampus. The next steps for this study are to

understand which proteins are significantly altered within the cortical and thalamic sections of these *Grn*-KO mice at 12 months, and whether granulin subunits serve as a therapeutic or toxic agent.

CONCLUSION

This study is the first to inject *Grn*-KO mice *in vivo* model of FTD with individual granulin subunits via AAV injections. Using this methodology, we were able to show that the addition of *Grn* subunits were able to rescue some markers of FTD such as lysosomal dysfunction as indicated by levels of Gal-3 and gliosis as indicated by GFAP. We were also able to prove that the injection of granulin subunits does not exacerbate known phenotypes of lysosomal dysfunction, gliosis, or inflammation within the hippocampus of FTD-like mice. In fact, this study is the first *in vivo* evidence to suggest that granulin subunits can be used as a treatment for FTD.

To further our understanding of the implications of AAV-GRN injections in the hippocampus, next steps include staining the hippocampal sections. This will inform us on the location of targeted proteins which could be beneficial because though in 12-month *Grn*-KO mice levels of CatZ and CatB did not change, the location of these proteins could have altered which could also indicate lysosomal dysfunction. Staining would also be another modality to confirm the findings of this study.

To better understand how AAV-GRN injections are impacting the brain as a whole, next steps include examining the same targets in cortical and thalamic sections. As FTD affects patients' behavior and personality, we may see more significant differences in lysosomal function, gliosis, and inflammation in the cortical sections of the 12-month-old *Grn*-KO mice. By conducting immunoblots of these cortical and thalamic

sections, we are able to use a targeted approach to examine how AAV-GRN injections affect different areas of the brain in an FTD-like mouse model. Prior studies used staining to show that the thalamus experienced the most robust change in FTD-like mice. Thus, for the thalamic sections, we will also be conducting proteomics analyses to examine in an unbiased manner how protein expression is affected within the thalamus and measure whether they are impacted by AAV-GRN injections.

If able, I would like to examine a similar set of targets used in this study in 18-month old *Grn*-KO mice. With time, I hypothesize that there would be more differences between the WT and KO mice, and therefore; we would be better able to measure if AAV-GRN subunits were able to rescue disease phenotypes. To do this, we would follow the same protocol as this study, but wait to sacrifice mice until 18 months after injection. Then, using immunoblotting, we would measure lysosomal dysfunction, gliosis, and inflammation of the hippocampal, cortical, and thalamic sections of the brain. In addition to these targets, I would also like to conduct an enzyme activity assay targeting Cathepsin D, which was shown to be trending downwards in the KO mice. An enzyme activity assay could be informative because though in this study, we found that certain levels of proteins did not change between the WT and KO genotypes, this does not exclude the possibility that enzyme activity did change. An enzyme activity assay would further our understanding on how the function of these crucial lysoosmal enzymes are affected in FTD-like mice.

Ultimately, this study found the first evidence that granulin subunits are beneficial in an in vivo model of FTD. This furthers our understanding of individual granulin subunit function and opens up novel treatment pathways for FTD.

Supplemental Tables and Figures

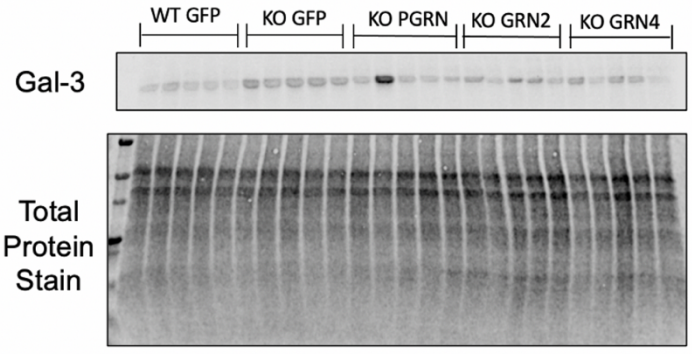
Resource Table (Supplemental Table 1)

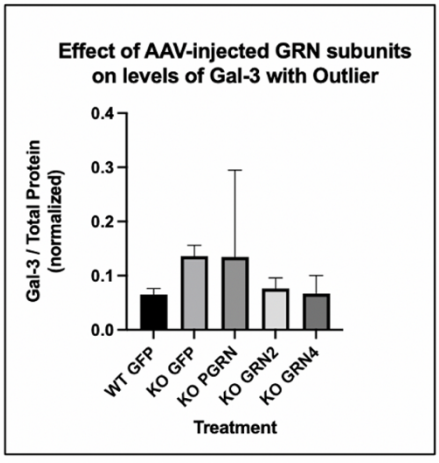
Protein	Host	Company	Concentration
Cathepsin B (CatB)	Goat	R&D AF965	0.1 microg/mL
Cathepsin D (CatD)	Goat	R&D AF1029	0.1 microg/mL
Cathepsin Z (CatZ)	Goat	Invitrogen PA5-47048	0.1 microg/mL
Cluster of differentiation 68 (CD68)	Rabbit	Abcam ab201340	0.5 microg/mL
Galectin-3 (Gal-3)	Goat	R&D AF1197	0.1 microg/mL
Glial fibrillary acidic protein (GFAP)	Rabbit	Dako Z0334	0.2 microg/mL
Glyceraldehyde -3 phosphate dehydrogenase (GAPDH)	Rabbit	CST 21185	0.1 microg/mL
Histone 3 (H3)	Rabbit	CST 4499P	0.1 microg/mL
Ionized calcium-binding adapter molecule 1 (IBA1)	Rabbit	CST 17198	0.1 microg/mL
Lysosomal associated membrane protein 1 (LAMP1)	Rabbit	CST 9091S	0.1 microg/mL
Subunit c of	Rabbit	Abcam	0.6 microg/mL

mitochondrial ATP synthase (SCMAS)		ab181243	
IR dye 800CW	Donkey	Licor 925-32214	0.5 microg/mL
IR dye 800CW	Donkey	Licor 926-32213	0.05 microg/mL
HRP	Goat	CST 7074P2	0.5 microg/mL
Alexa Fluor +800	Donkey	Invitrogen A32808	1 microg/mL
Alexa Fluor +680	Donkey	Invitrogen A32802	1 microg/mL
Alexa Fluor +680	Donkey	Invitrogen A32860	1 microg/mL

Supplemental Figure 1

Immunoblot and bar graph of Gal-3 levels in all treatment groups with outlier included.





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