Progranulin Gene Therapy Improves Lysosomal Dysfunction and Microglial Pathology Associated with Frontotemporal Dementia and Neuronal Ceroid Lipofuscinosis

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Loss-of-function mutations in progranulin, a lysosomal glycoprotein, cause neurodegenerative disease. Progranulin haploinsufficiency causes frontotemporal dementia (FTD) and complete progranulin deficiency causes CLN11 neuronal ceroid lipofuscinosis (NCL). Progranulin replacement is a rational therapeutic strategy for these disorders, but there are critical unresolved mechanistic questions about a progranulin gene therapy approach, including its potential to reverse existing pathology. Here, we address these issues using an AAV vector (AAV-Grn) to deliver progranulin in Grn−/− mice (both male and female), which model aspects of NCL and FTD pathology, developing lysosomal dysfunction, lipofuscinosis, and microgliosis. We first tested whether AAV-Grn could improve preexisting pathology. Even with treatment after onset of pathology, AAV-Grn reduced lipofuscinosis in several brain regions of Grn−/− mice. AAV-Grn also reduced microgliosis in brain regions distant from the injection site. AAV-expressed progranulin was only detected in neurons, not in microglia, indicating that the microglial activation in progranulin deficiency can be improved by targeting neurons and thus may be driven at least in part by neuronal dysfunction. Even areas with sparse transduction and almost undetectable progranulin showed improvement, indicating that low-level replacement may be sufficiently effective. The beneficial effects of AAV-Grn did not require progranulin binding to sortilin. Finally, we tested whether AAV-Grn improved lysosomal function. AAV-derived progranulin was delivered to the lysosome, ameliorated the accumulation of LAMP-1 in Grn−/− mice, and corrected abnormal cathepsin D activity. These data shed light on progranulin biology and support progranulin-boosting therapies for NCL and FTD due to GRN mutations.

Key words: cathepsin D; frontotemporal dementia; gene therapy; lysosome; neuronal ceroid lipofuscinosis; progranulin

Significance Statement

Heterozygous loss-of-function progranulin (GRN) mutations cause frontotemporal dementia (FTD) and homozygous mutations cause neuronal ceroid lipofuscinosis (NCL). Here, we address several mechanistic questions about the potential of progranulin gene therapy for these disorders. GRN mutation carriers with NCL or FTD exhibit lipofuscinosis and Grn−/− mouse models develop a similar pathology. AAV-mediated progranulin delivery reduced lipofuscinosis in Grn−/− mice even after the onset of pathology. AAV delivered progranulin only to neurons, not microglia, but improved microgliosis in several brain regions, indicating cross talk between neuronal and microglial pathology. Its beneficial effects were sortilin independent. AAV-derived progranulin was delivered to lysosomes and corrected lysosomal abnormalities. These data provide in vivo support for the efficacy of progranulin-boosting therapies for FTD and NCL.

Introduction

Loss-of-function mutations in progranulin (GRN) cause neurodegenerative disease with a gene-dose effect. GRN mutations are among the leading causes of dominantly inherited frontotemporal dementia (FTD) (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006). These GRN mutations typically cause progranulin hap-
loinsufficiency, with plasma progranulin levels reduced by >50% relative to controls (Finch et al., 2009). Individuals with mutations in both GRN alleles, resulting in nearly complete progranulin deficiency, develop the lysosomal storage disorder neuronal ceroid lipofuscinosis (NCL) (Smith et al., 2012; Canafoglia et al., 2014; Almeida et al., 2016). NCL due to GRN mutations has been termed CLN11-NCL and is characterized by seizures and retinal degeneration with onset in the early twenties (Smith et al., 2012; Canafoglia et al., 2014; Almeida et al., 2016). Despite very different clinical presentations, FTD patients with GRN mutations exhibit some pathological similarities to CLN11-NCL because brains of patients with both diseases exhibit gliosis, increased levels of lysosomal proteins, and lipofuscinosis (Götzel et al., 2014; Ward et al., 2017).

Progranulin is a widely expressed, secreted glycoprotein that performs a variety of functions (Bateman and Bennett, 1998; Erikson and Mackenzie, 2008; Cenik et al., 2012; Nguyen et al., 2013). Progranulin acts as a trophic factor for many cell types, including neurons (Van Damme et al., 2008; Ryan et al., 2009; Gass et al., 2012; Beel et al., 2017). It also modulates inflammation and facilitates wound healing (Zhu et al., 2002; Yin et al., 2010). The development of NCL in patients with complete progranulin deficiency shows that progranulin is critical for proper lysosomal function (Smith et al., 2012). Recent data have shown that progranulin is critical for proper trafficking and function of lysosomal enzymes such as β-glucocerebrosidase and cathepsin D (CatD), providing a potential mechanism by which progranulin promotes lysosomal function (Jian et al., 2016; Beel et al., 2017; Valdez et al., 2017; Zhou et al., 2017b). Progranulin knock-out mice have been used to study the effects of progranulin insufficiency and model the gene-dose effect seen in humans. GRN+/− mice, but not GRN−/− mice, develop NCL-like pathology, including accumulation of lipofuscin and lysosomal proteins in the brain, astrogliosis, and microgliosis (Ahmed et al., 2010; Smith et al., 2012; Wils et al., 2012; Filiano et al., 2013; Tanaka et al., 2014).

Because most, if not all, disease-associated GRN mutations appear to be loss-of-function mutations, boosting progranulin levels, particularly in the brain, is a rational approach to preventing or treating FTD and NCL in GRN mutation carriers. Several progranulin-boosting approaches have been developed to increase progranulin levels from the intact GRN allele in heterozygous mutation carriers (Hu et al., 2010; Capell et al., 2011; Cenik et al., 2011; Lee et al., 2014). However, these approaches are not suitable for CLN11-NCL patients, who have mutations in both GRN alleles (Smith et al., 2012; Canafoglia et al., 2014; Almeida et al., 2016). For these CLN11-NCL patients, a gene therapy approach would be required to express progranulin. Viral vectors are an excellent tool for such an approach and have been tested in models of CLN1-NCL, CLN2-NCL, and CLNS-NCL (Grieffey et al., 2006; Passini et al., 2006; Cabrera-Salazar et al., 2007; Sondhi et al., 2007; Maculey et al., 2012; Hughes et al., 2014; Katz et al., 2015). In addition, we have recently observed that restoration of progranulin with an adeno-associated virus (AAV) vector to GRN+/− mice corrects social behavior deficits (Arrant et al., 2017).

The goal of this study was to determine whether AAV-mediated expression of progranulin could improve NCL-like pathology in GRN−/− mice when administered after the onset of pathology and to elucidate mechanisms by which progranulin gene therapy might work. We infused an AAV vector expressing mouse progranulin (AAV-Grn) or an AAV-GFP control virus into the medial prefrontal cortex (mPFC) of wild-type or GRN−/− mice after the onset of pathology and collected brain tissue for analysis 8–10 weeks later.

### Materials and Methods

**Animals.** The line of progranulin-deficient mice used for this study was generated and crossed onto a C57BL/6 background as described previously (Martens et al., 2012; Filiano et al., 2013). The mice used for this study were obtained by breeding Grn+/− mice to produce wild-type, Grn+/−, and Grn−/− mice. The resulting wild-type and Grn−/− littermates were used for all experiments. Male and female mice were included in the study. The mice were bred and housed in a barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were maintained on a 12 h:12 h light/dark cycle with lights on at 6:00 A.M. and were given ad libitum access to food (NIH-31 diet #7917; Envigo) and water in all phases of the study. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

**Antibodies.** The following antibodies were used for immunostaining and Western blot: CD68 (1:500 rat monoclonal, #MCA1957; Bio-Rad), Iba1 (1:500 rabbit polyclonal, #019-19741; Wako), MHCI:1 (100 mouse monoclonal, #14-5321-81; Thermo Fisher Scientific), GFAP (1:1000 rabbit polyclonal, #20334; Dako), NeuN (1:1000 mouse monoclonal, #MAB377; Millipore/Sigma-Aldrich), progranulin (1:500 for Western blot and chromogenic immunostaining, 1:200 for fluorescent immunostaining, sheep polyclonal, #AF2557; R&D Systems), CatD (1:500 goat polyclonal, #sc-6486; Santa Cruz Biotechnology), LAMP-1 (1:250 rat monoclonal, #1D4B; Developmental Studies Hybridoma Bank), sortilin (1:1000 rabbit polyclonal, #ab16640; Abcam), α-tubulin (1:1000 mouse monoclonal, #T5168; Sigma-Aldrich), and SCMAS (1:300 rabbit polyclonal, provided by Dr. Yasuo Uchiyama, Juntendo University) (Koike et al., 2000).

**AAV constructs and vectors.** An AAV2/1 progranulin vector (AAV-Grn, rAAV2-CBA-mGrn-Myc-WPRE-RB) and GFP-expressing control vector (AAV-GFP, #AV-1-PV1963, AAV1-CB7-CI-eGFP-WPRE-RB) were produced at the University of Pennsylvania Vector Core as described previously (Arrant et al., 2017). An N-terminal-tagged mouse progranulin AAV construct was also generated to serve as a positive control for sortilin immunoprecipitation. This N-terminal-tagged progranulin AAV construct was generated using a synthetic construct containing the RNA-coding sequence of mouse progranulin with an HA tag inserted after the signal peptide (GenScript). The N-terminal HA-tagged mouse progranulin sequence was then the same CIGW AAV2 vector used for the C-terminal myc-tagged progranulin vector to generate a similar N-terminal tagged mouse progranulin AAV construct (rAAV2-CBA-HA-mGrn-WPRE-RB) (St Martin et al., 2007; Arrant et al., 2017).

**AAV injection.** AAV-Grn or AAV-GFP were bilaterally infused into the mPFC of 10–12-month-old wild-type and Grn−/− mice using stereotaxic surgery (coordinates +1.9 mm anterior and ±0.3 mm lateral from bregma, −2.2 mm from the surface of the skull) under isoflurane anesthesia as described previously (Arrant et al., 2017). Then, 1 μL of AAV (7.36 × 1011 genomes/ml) was infused into each hemisphere with a syringe pump (Harvard Apparatus) at a flow rate of 0.5 μl/min. After allowing 5 min for diffusion into the tissue, the injection needle (Hamilton) was withdrawn, the skull was sealed with bone wax, and the wound was closed with surgical staples.

**Brain and plasma collection.** Mice were killed for collection of tissue samples 8–10 weeks after AAV injection. The mice were anesthetized with pentobarbital (100 mg/kg, Fatal Plus; Vortech Pharmaceuticals) and blood was collected by cardiac puncture in syringes containing EDTA (250 μl) to prevent clotting. The blood was kept on ice and later centrifuged at 5000 × g for 10 min at 4°C to separate plasma. The mice were then transcardially perfused with 0.9% saline. Brains were removed and bisected into hemibrains, one of which was immediately frozen on dry ice for biochemical analysis and the other postfixed for 48 h in 4% paraformaldehyde for histological analysis.
**Immunostaining.** Fixed hemibrains were cryoprotected in 30% sucrose and cut into 30 μm sections on a sliding microtome (Leica Biosystems). The sections were then immunostained as described previously (Palop et al., 2011). For analysis of pathology and a qualitative assessment of progranulin immunoreactivity, the sections were incubated overnight in primary antibody and the following day were incubated with a species-matched biotinylated secondary antibody (Vector Laboratories), followed by avidin-biotin complex (Vectastain Elite; Vector Laboratories). Immunostaining was visualized with diaminobenzidine (MP Biomedical).

For immunofluorescence, brain sections were sequentially immunostained for progranulin followed by an Alexa Fluor-488-conjugated anti-sheep antibody (Thermo Fisher Scientific), and then markers for neurons (NeuN) or microglia (Iba1) followed by species-matched Alexa Fluor-647-conjugated antibodies (Thermo Fisher Scientific). Subcellular localization of progranulin within neurons was assessed by sequential immunostaining for progranulin, NeuN, and LAMP-1, followed by species-matched Alexa Fluor-488, Alexa Fluor-647, and Alexa Fluor-594 antibodies (Thermo Fisher Scientific).

**Microscopy and image analysis.** For pathology analysis, immunostained sections were imaged at 20× with a light microscope (Nikon) and CCD camera (Nikon). Low-magnification, high-resolution images of progranulin immunostaining were obtained with a slide scanner (Path-Scan Enabler IV; Meyer Instruments). Lipofuscinosis was measured by mounting unstrained tissue sections on slides and coveringslipping with a mounting medium containing DAPI (Vectorshield; Vector Laboratories).

**CatD activity assay.** CatD activity was determined by incubating brain tissue lysates with a fluorogenic CatD/E substrate (Yasuda et al., 1999). Brain tissue was homogenized in lysis buffer without protease inhibitors (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) and centrifuged at 5000 × g for 10 min. The protein concentration of the supernatant was determined by Bradford assay (Bio-Rad Laboratories) and recorded with a NanoDrop 1000 spectrophotometer. The protein concentration of the supernatant was determined by Bradford assay (Bio-Rad Laboratories) and recorded with a NanoDrop 1000 spectrophotometer. The protein concentration of the supernatant was determined by Bradford assay (Bio-Rad Laboratories) and recorded with a NanoDrop 1000 spectrophotometer. The protein concentration of the supernatant was determined by Bradford assay (Bio-Rad Laboratories) and recorded with a NanoDrop 1000 spectrophotometer. The protein concentration of the supernatant was determined by Bradford assay (Bio-Rad Laboratories) and recorded with a NanoDrop 1000 spectrophotometer.
A key question for progranulin-boosting therapies in NCL and FTD is whether boosting progranulin after the onset of pathology could reduce lipofuscinosis and microgliosis. To address this question, we treated wild-type and Grn−/− mice with an AAV vector expressing mouse progranulin with a C-terminal myc tag (AAV-Grn) or a control AAV-GFP vector at age 10–12 months. Grn−/− mice exhibit robust NCL-like pathology at this age, characterized by lipofuscin accumulation and gliosis (Ahmed et al., 2010; Wils et al., 2012; Filiano et al., 2013; Petkau et al., 2016). The AAV vectors were intracranially injected into the mPFC, as in our prior study investigating the behavioral effects of AAV-Grn in Grn−/− mice (Arrant et al., 2017). A small additional group of uninjected Grn−/− mice was included in the study as a positive control for the expected abnormalities in Grn−/− mice to rule out nonspecific effects of the control AAV-GFP treatment.

Regional distribution of progranulin expression

We first characterized the pattern of progranulin expression produced by AAV-Grn. In a previous study, we demonstrated that this AAV-Grn vector strongly increased progranulin levels in wild-type and Grn+/+ mice at the injection site in the mPFC, with smaller but significant increases in progranulin throughout the forebrain (Arrant et al., 2017). To determine whether AAV-Grn had similar effects in Grn−/− mice, we qualitatively screened AAV-mediated progranulin expression by immunohistochemistry (Fig. 1a,b) and measured progranulin protein levels by ELISA (Fig. 1c). As expected, progranulin immunoreactivity was strongly increased in the mPFC of wild-type and Grn−/− mice treated with AAV-Grn, and more modestly increased in the septum and medial regions of the striatum and thalamus (Fig. 1a). With high-magnification imaging, we observed large numbers of strongly progranulin-immunoreactive cells in the mPFC (Fig. 1b). Brain regions more distal to the injection site, such as the ventral postero medial/lateral (VP/MPL) thalamus and CA3 of the hippocampus, had scattered strongly progranulin-immunoreactive cells, but far fewer than mPFC (Fig. 1b). Global analysis of progranulin levels by ELISA (Fig. 1c) revealed that AAV-Grn increased progranulin (RM-ANOVA: effect of virus, F(3,8) = 49.78, p < 0.001) in a genotype-specific (RM-ANOVA: genotype × virus, F(1,10) = 27.7, p = 0.0004) and region-specific (RM-ANOVA: region × virus, F(3,36) = 15.3, p = 0.0011, region × genotype × virus, F(3,56) = 8.183, p = 0.008) manner. In Grn+/+ mice, AAV-Grn increased progranulin in all brain regions examined (Fig. 1c). In Grn−/− mice, progranulin levels induced by AAV-Grn were lower than in Grn+/+ mice as measured by ELISA. In fact, the scattered progranulin-positive cells observed by immunohistochemistry in thalamus and hippocampus (Fig. 1b, bottom right) were not sufficient to yield a significant progranulin signal by ELISA (Fig. 1c). In this case, due to the low number of transduced cells, immunostaining was the more sensitive method of detection. We conclude that AAV-Grn-treated Grn−/− mice expressed lower levels of AAV-mediated progranulin than AAV-Grn-treated wild-type mice for the reasons discussed below. The lower progranulin levels in Grn−/− mice were specific to AAV-Grn because levels of GFP expression induced by AAV-GFP did not differ between Grn+/+ and Grn−/− mice (Fig. 1d).

We suspected that the scattered progranulin-positive cells in distal regions represented cells transduced by AAV-Grn spreading from the injection site; the alternative possibility is that these cells had taken up progranulin secreted from the axons of transduced cortical neurons (Petkovsek et al., 2013). To distinguish between these possibilities, we measured Grn RNA levels. We observed a definite increase in Grn RNA at the mPFC injection site and a much more modest but significant increase in Grn RNA in the thalamus of AAV-Grn-treated mice (Fig. 1e). As with progranulin protein levels, AAV-Grn-treated Grn−/− mice exhibited detectable increases in Grn RNA, but Grn RNA levels were
lower than in AAV-Grn-treated wild-type mice. Based on these data, we conclude that AAV-Grn transduced small numbers of cells in regions distant from the mPFC, probably due to viral particles diffusing through the brain to a limited extent.

**AAV-Grn transduces neurons, but not microglia**

We next investigated which cell types were transduced by AAV-Grn. In our prior study with AAV-Grn in wild-type and Grn−/− mice, we found that AAV-Grn transduced neurons, but not...
microglia (Arrant et al., 2017). To confirm that AAV-Grn has similar effects in Grn<sup>−/−</sup> mice, we performed double immunostaining for progranulin and markers of neurons (NeuN) and microglia (Iba1) in AAV-Grn-treated Grn<sup>−/−</sup> mice (Fig. 2a,b) to determine whether microglia exhibited any progranulin immunoreactivity. Because this immunostaining was performed in Grn<sup>−/−</sup> mice, progranulin immunoreactivity was entirely composed of AAV-expressed progranulin. All of the strongly progranulin-immunoreactive cells observed were NeuN positive (Fig. 2a); no Iba1-positive cells exhibited progranulin immunoreactivity (Fig. 2b). Therefore, AAV-Grn only transduced neurons in Grn<sup>−/−</sup> mice, consistent with our prior study in wild-type and Grn<sup>+/−</sup> mice (Arrant et al., 2017) and the expected tropism of the AAV. The absence of progranulin from microglia is somewhat surprising because progranulin secreted from neurons could potentially be taken up by microglia. Although we do not have in vivo data on the secretion of AAV-expressed progranulin, cultured cells transfected with the AAV-Grn plasmid secrete myc-tagged progranulin (data not shown), indicating that AAV-expressed progranulin can be secreted by some cell types. Therefore, the AAV-expressed progranulin produced in neurons in vivo is either not secreted, not taken up by microglia, or is rapidly degraded within microglia.

**AAV-Grn improves lipofuscinosis in Grn<sup>−/−</sup> mice**

We tested the effects of AAV-Grn on lipofuscinosis by quantifying autofluorescence in the mPFC, motor cortex, hippocampus (CA3), and VPM/VPL thalamus of AAV-treated wild-type and Grn<sup>−/−</sup> mice (Fig. 3a,b,d). There was a significant effect of AAV that was dependent on genotype (three-way RM-ANOVA across all brain regions: genotype × virus, F(1,28) = 10.09, p = 0.0035). Because effects were also brain-region dependent (region × genotype, F(3,27) = 7.845, p = 0.0006), we performed separate analyses of each brain region. AAV-Grn significantly reduced lipofuscinosis in the CA3 and thalamus of Grn<sup>−/−</sup> mice (Fig. 3a,b,d). There were similar trends for reduction of lipofuscinosis in the mPFC (ANOVA: genotype × virus, F(1,30) = 3.902, p = 0.0575) and motor cortex (ANOVA: genotype × virus, F(1,31) = 3.43, p = 0.0736) of AAV-Grn-treated Grn<sup>−/−</sup> mice. These data show a therapeutic benefit of progranulin restoration to Grn<sup>−/−</sup> mice even after the onset of lipofuscinosis. The significant improvement in lipofuscinosis in the hippocampus and thalamus of AAV-Grn-treated Grn<sup>−/−</sup> mice, despite the very modest increase in progranulin in these brain regions (Fig. 1), indicates that the progranulin from small numbers of transduced cells is sufficient to produce beneficial effects. The fact that reduction of lipofuscinosis did not reach significance in the mPFC (p = 0.0575) or motor cortex (p = 0.0736) could be due to a floor effect because these regions have less lipofuscin accumulation than the hippocampus and thalamus.

As an additional measure of lipofuscinosis, we performed immunostaining for subunit C of mitochondrial ATP synthase (SCMAS), a protein component of lipofuscin (Hall et al., 1991; Kominami et al., 1992; Elleder et al., 1997) that accumulates in Grn<sup>−/−</sup> mice (Götzl et al., 2014; Zhou et al., 2017a). As expected, Grn<sup>−/−</sup> mice exhibited a striking increase in SCMAS immunoreactivity (Fig. 3c,e). AAV-Grn reduced SCMAS immunoreactivity in Grn<sup>−/−</sup> mice, providing further support for a reduction in lipofuscinosis in AAV-Grn-treated Grn<sup>−/−</sup> mice.

**AAV-Grn improves microgliosis away from the injection site in the mPFC**

In addition to lipofuscinosis, the pathology of Grn<sup>−/−</sup> mice is characterized by microgliosis (Ahmed et al., 2010; Wils et al., 2012; Filiano et al., 2013; Tanaka et al., 2014; Arrant et al., 2015; Petkau et al., 2016). To determine the effects of AAV-Grn on microgliosis, we measured CD68 immunoreactivity in AAV-GFP- and AAV-Grn-treated wild-type and Grn<sup>−/−</sup> mice. Global analysis across all brain regions indicated an effect of AAV-Grn on microgliosis that varied by brain region (RM-ANOVA region × genotype × virus, F(3,26) = 3.03, p = 0.0473; region × genotype, F(3,26) = 13.51, p < 0.0001). CD68 immunoreactivity was significantly reduced in motor cortex and CA3 of AAV-Grn-treated Grn<sup>−/−</sup> mice relative to their GFP-treated controls (Fig. 4a,b). As a further analysis, we measured the soma size of Iba1-positive cells. Grn<sup>−/−</sup> mice exhibit abnormal microglial morphology as they
age, with Iba1-positive microglia exhibiting less ramification and larger soma size, indicative of activation (Ahmed et al., 2010; Karperien et al., 2013). Iba1-positive soma size was increased in Grn/H11002/H11002 mice in the VPM/VPL thalamus, consistent with a prior report (Fig. 4c,d) (Ahmed et al., 2010). AAV-Grn significantly reduced Iba1-positive soma size, partially correcting Iba1-positive cell morphology (Fig. 4c,d). Together, the CD68 and Iba1 analyses indicate that AAV-Grn reduced microgliosis in brain regions away from the injection site, including motor cortex, CA3, and VPM/VPL thalamus. This improvement in microgliosis in the absence of detectable increases in microglial progranulin (Fig. 2b) has several potential interpretations. Microgliosis could be reactive to neuronal dysfunction and indirectly improved by amelioration of neuronal dysfunction by neuronal progranulin restoration.

Alternatively, AAV-expressed progranulin secreted from neurons could reduce microgliosis through effects on microglial cell surface receptors or could be taken up and very rapidly degraded by microglia.

AAV-Grn causes localized injection site inflammation in Grn−/− mice and a nonspecific reaction

In an unexpected contrast to regions away from the injection site, AAV-Grn strongly increased microglial markers near the injection site in the mPFC in Grn−/− mice, but not in wild-type mice. Both CD68 and Iba1 immunoreactivity were strongly increased in mPFC in AAV-Grn-treated Grn−/− mice, but not in AAV-Grn-treated wild-type mice (Fig. 5a–e). Iba1-positive cell size was not significantly affected by AAV-Grn in the mPFC of Grn−/− mice.
mice (data not shown), although there was a difference in staining pattern, with more diffuse Iba1 labeling and more Iba1-positive cells (Fig. 5a). To determine whether these increases in microglial markers reflected immune activation, we immunostained for MHCII, which is expressed by activated microglia during inflammation (Lynch, 2009; Franco and Fernández-Suárez, 2015). There was an increase in MHCII immunoreactivity in Grn<sup>−/−</sup> mice (RM-ANOVA effect of genotype, \( F_{1,21} = 7.471, p = 0.0125 \)), which was driven by very high expression of MHCII in the mPFC of AAV-Grn-treated Grn<sup>−/−</sup> mice, indicating local inflammation (Fig. 5a,d). In the motor cortex, CA3, and VPM/VPL thalamus of AAV-Grn-treated Grn<sup>−/−</sup> mice, MHCII staining was not significantly elevated upon statistical analysis. These data indicate a strong local inflammatory response to AAV-Grn in Grn<sup>−/−</sup> mice, but not in wild-type mice. Importantly, Grn<sup>−/−</sup> mice did not have a similar reaction to AAV-GFP, indicating that this local inflammation was not a general response to AAV injection.

In considering the opposing, region-specific effects of AAV-Grn on microgliosis in Grn<sup>−/−</sup> mice (improvement of microglial markers), it is important to note that Grn<sup>−/−</sup> mice did not have a similar reaction to AAV-GFP, indicating that this local inflammation was not a general response to AAV injection.
Gliosis in multiple regions except at the mPFC injection site, where it was worse), we hypothesized that Grn−/− mice may have reacted to the exogenous progranulin as a nonself protein, given the complete absence of progranulin throughout their lifespan. In support of this possibility, wild-type mice (Fig. 5) and Grn+/− mice (data not shown) did not exhibit microgliosis or increased MHCII expression at the AAV-Grn injection site. In addition, antigen-presenting cells use MHC-II to present antigens to T cells, indicating a cell-mediated immune response to AAV-expressed progranulin in Grn−/− mice (Roche and Furuta, 2015). Furthermore, previous studies have shown that expressing foreign proteins with AAV vectors can induce nonself reactions that resemble the effects of AAV-Grn in Grn−/− mice when using AAV serotypes capable of transducing antigen-presenting cells (Ciesielska et al., 2013; Samaranch et al., 2014). We hypothesized that a similar mechanism could be at work in our study, in which...
*Grn*−/− mice have a large immune response to progranulin, a secreted protein, but a very small response to GFP, which, although it is a foreign protein, should remain primarily intracellular. Therefore, progranulin secreted from AAV-Grn-transduced neurons would be readily accessible to antigen-presenting cells, whereas GFP from AAV-GFP-transduced neurons would not.

To determine whether *Grn*−/− mice had a nonself reaction to progranulin and GFP, we tested for progranulin and GFP antibodies in the plasma of AAV-treated mice. We ran recombinant mouse progranulin or recombinant GFP from *Aequorea victoria* on SDS-PAGE and transferred the protein to PVDF membranes, and then probed the membranes with plasma isolated from individual mice. An anti-mouse secondary antibody was used to determine whether circulating antibodies were bound to the recombinant proteins. For progranulin, we tested plasma from wild-type mice treated with AAV-Grn and *Grn*−/− mice treated with AAV-GFP or AAV-Grn. Of these groups, only AAV-Grn-treated *Grn*−/− mice had circulating anti-mouse progranulin antibodies (Fig. 6a). Of the nine AAV-Grn-treated *Grn*−/− mice tested, eight were positive for circulating anti-mouse progranulin antibodies (Fig. 6b), suggesting that AAV-Grn induced a nonself reaction in these mice. This effect is probably unique to the *Grn*−/− mouse model and is unlikely to be an issue in patients with *GRN* mutations, who still express some progranulin and so are not at risk of nonself recognition.

In testing for GFP antibodies, we analyzed plasma from wild-type and *Grn*−/− mice treated with AAV-GFP, and from mice that received no AAV. Plasma from both wild-type and *Grn*−/− mice treated with AAV-GFP detected recombinant GFP, indicating the presence of anti-GFP antibodies (see Fig. 9c). The lack of genotype specificity is unsurprising as GFP is a foreign protein for both genotypes. However, the presence of detectable anti-GFP antibodies was somewhat surprising given the lack of a similar inflammatory response as observed in AAV-Grn-treated *Grn*−/− mice. Given our hypothesis that GFP should be less likely to generate an immune response than progranulin due to GFP’s almost exclusively intracellular location, we hypothesized that AAV-GFP-treated wild-type and *Grn*−/− mice might have
mounted a much weaker nonself response to GFP than AAV-Grn-treated Grn$^{-/-}$ mice generated to progranulin. To test this hypothesis, we analyzed anti-GFP and anti-progranulin antibody titers by progressively diluting the plasma from AAV-GFP-treated wild-type and Grn$^{-/-}$ mice and AAV-Grn-treated Grn$^{-/-}$ mice and determining the maximum dilution at which immunoreactivity was maintained. The anti-progranulin antibodies generated by AAV-Grn-treated Grn$^{-/-}$ mice were still detectable at plasma dilutions up to 1:2500, whereas the anti-GFP antibodies generated by AAV-GFP-treated wild-type and Grn$^{-/-}$ mice were detectable at plasma dilutions of 1:100–1:1500 (Fig. 5c–d). These data indicate greater levels of anti-progranulin antibodies in AAV-Grn-treated Grn$^{-/-}$ mice than of anti-GFP antibodies in AAV-GFP-treated mice, which, along with the greater inflammation in AAV-Grn-treated Grn$^{-/-}$ mice, is consistent with a stronger nonself reaction to progranulin than to GFP.

To determine whether the mPFC inflammation induced by AAV-Grn in Grn$^{-/-}$ mice had negative functional effects, we tested the mice in two mPFC-dependent behavioral assays: spontaneous alternation in the Y-maze (Yang et al., 2014) and extinction of conditioned fear (Giustino and Marem, 2015). We observed no deficits in either Y-maze spontaneous alternation (Fig. 5e) or conditioned fear extinction (Fig. 5f) in AAV-Grn-treated Grn$^{-/-}$ mice relative to AAV-GFP-treated Grn$^{-/-}$ mice or uninjected Grn$^{-/-}$ mice. Therefore, the inflammation in the mPFC was not sufficient to produce detectable mPFC-dependent behavioral impairments in Grn$^{-/-}$ mice. These data are consistent with our prior study showing that AAV-Grn does not disrupt mPFC-dependent behaviors in wild-type or Grn$^{+/+}$ mice (Arrant et al., 2017).

**Effects of AAV-Grn are independent of sortilin binding**

Having observed that AAV-Grn reduced lipofuscinosis and microgliosis in Grn$^{-/-}$ mice (Figs. 3, 4), we began to investigate the mechanisms by which progranulin mediated these beneficial effects. First, we determined whether the beneficial effects of progranulin gene therapy with AAV-Grn depend on progranulin binding to sortilin, a major progranulin receptor (Hu et al., 2010). The C terminus of progranulin is critical for sortilin binding and C-terminal tags on progranulin have been reported to disrupt sortilin binding (Zheng et al., 2011). We therefore assessed whether our C-terminal myc-tagged progranulin binds sortilin. Consistent with prior reports, the C-terminal myc tag on our AAV-Grn construct almost completely eliminated sortilin binding (Fig. 7). Therefore, the improvement in pathology by AAV-Grn that we observed does not require sortilin binding.

**AAV-expressed progranulin improves lysosomal function**

Lipofuscin consists of undegraded lysosomal storage material, so it is likely that the reduction in lipofuscinosis in AAV-Grn-treated Grn$^{-/-}$ mice was due to improved lysosomal function. To test this hypothesis, we assessed CatD levels and activity, as well as levels of the lysosomal membrane protein LAMP-1. CatD expression is increased in the brain of Grn$^{-/-}$ mice at ages ranging from 16–24 months and recent studies have shown that progranulin binds to CatD and may regulate its activity (Wils et al., 2012; Götzl et al., 2014; Beel et al., 2017; Valdez et al., 2017; Zhou et al., 2017b). This effect is age dependent because a recent study of 2-month-old Grn$^{-/-}$ mice did not observe elevated CatD levels in the brain (Zhou et al., 2017b).

First, we investigated the frontal cortex of 2- to 3-month-old and 8- to 10-month-old wild-type and Grn$^{-/-}$ mice (Fig. 8) and observed increased CatD activity and protein levels in 8- to 10-month-old (Fig. 8e–g), but not 2- to 3-month-old (Fig. 8a–c) Grn$^{-/-}$ mice, consistent with prior reports on CatD protein levels and activity (Götzl et al., 2014; Beel et al., 2017; Zhou et al., 2017b). Others have reported that Grn$^{-/-}$ mice or cells actually have lower CatD activity when normalized to levels of mature CatD protein, suggesting a functional impairment of CatD in Grn$^{-/-}$ mice (Beel et al., 2017; Valdez et al., 2017). We assessed this “relative activity” by dividing the fluorescence generated at the final time point of the CatD activity assay by the relative levels of mature CatD. We found reduced relative CatD activity in 8- to 10-month-old Grn$^{-/-}$ mice (Fig. 8h), but not in 2- to 3-month-old Grn$^{-/-}$ mice (Fig. 8d), indicating that the impairments in relative CatD activity are also age dependent. LAMP-1 levels followed a similar time course and were significantly increased in 8- to 10-month-old Grn$^{-/-}$ mice (Fig. 8f), but not 2- to 3-month-old Grn$^{-/-}$ mice (Fig. 8b,c). The age-dependent increase in CatD and LAMP-1 levels likely reflects a compensatory increase in lysosomal biogenesis induced in response to the lysosomal dysfunction associated with progranulin deficiency.

A notable feature of progranulin-immunoreactive neurons (Fig. 2a) in AAV-Grn-treated Grn$^{-/-}$ mice was the presence of very bright puncta within each cell that resembled vesicular immunolabeling. Endogenous progranulin strongly localizes to lysosomes, so we performed triple immunostaining for progranulin, NeuN, and LAMP-1, a lysosomal membrane protein, to determine whether these bright progranulin puncta represented lysosomal progranulin. We observed strong colocalization of progranulin and LAMP-1 (Fig. 9a), showing that AAV-expressed progranulin is transported to lysosomes in neurons, placing it in the proper cellular context to improve lipofuscinosis (Fig. 3). We then tested whether AAV-Grn would normalize CatD activity and protein levels in Grn$^{-/-}$ mice. We investigated the ventral striatum of AAV-treated wild-type and Grn$^{-/-}$ mice because the PFC and other brain regions were processed with protease inhibitors for progranulin ELISA (Fig. 5). As in other regions, CatD activity was increased in the ventral striatum of...
**Figure 8.** *Grn* 

<i>Grn</i><sup>−/−</sup> mice exhibit age-dependent increases in CatD and LAMP-1. <b>a</b>, CatD activity was not significantly increased at age 2–3 months (<i>n</i> = 6–7 mice per genotype) in the frontal cortex of 2- to 3-month-old *Grn* <sup>−/−</sup> mice. <b>b</b>, <i>c</i>, CatD and LAMP-1 protein levels were also not significantly increased at 2–3 months. <b>d</b>, In addition, the relative CatD activity of 2- to 3-month-old *Grn* <sup>−/−</sup> mice (fluorescence at 40 min/mature CatD) was not significantly different from wild-type. <b>e</b>, CatD activity was significantly increased in the frontal cortex of 8- to 10-month-old *Grn* <sup>−/−</sup> mice (<i>n</i> = 12–13 per genotype; activity, RM-ANOVA, genotype × time interaction, <i>F</i><sub>20,460</sub> = 30.11, <i>p</i> < 0.0001; *<i>p</i> < 0.05, **<i>p</i> < 0.01, ***<i>p</i> < 0.001, and ****<i>p</i> < 0.0001 versus wild-type, Fisher’s post hoc test). <b>f</b>, Both pro-CatD (<i>t</i><sub>23</sub> = 3.748, <i>p</i> = 0.0011) and mature CatD (<i>t</i><sub>23</sub> = 4.283, <i>p</i> = 0.0003) protein levels were also increased at 8–10 months (**<i>p</i> < 0.01 and ***<i>p</i> < 0.001, <i>t</i> test). LAMP-1 levels were also increased in the frontal cortex of 8- to 10-month-old *Grn* <sup>−/−</sup> mice (<i>t</i><sub>22</sub> = 4.942, <i>p</i> < 0.0001), indicating a more general increase in lysosomal proteins (****<i>p</i> < 0.0001 vs wild-type, <i>t</i> test). <b>h</b>, Relative CatD activity was decreased in 8- to 10-month-old *Grn* <sup>−/−</sup> mice (<i>t</i><sub>19</sub> = 2.231, <i>p</i> = 0.0379). Representative images of Western blots are shown in <b>c</b> and <b>g</b>. The CatD blots show bands for pro-CatD (<sup>P</sup>) and mature CatD (<sup>M</sup>).
Figure 9. AAV-Grn normalizes CatD activity in Grn−/− mice. a, Progranulin-immunoreactive neurons exhibited bright puncta that were generally colabeled with the lysosomal marker LAMP-1 (white arrows), indicating lysosomal localization of progranulin. b, AAV-GFP-treated Grn−/− mice had elevated CatD activity in the ventral striatum (n = 6–11 mice per group, 3-way RM-ANOVA effect of genotype, F[1,31] = 12.14, p = 0.0015), which was normalized by AAV-Grn treatment (3-way RM-ANOVA effect of virus, F[1,31] = 7.47, p = 0.0104, ‡2-way RM-ANOVA of AAV-GFP-treated vs AAV-Grn-treated Grn−/− mice: virus × time interaction, F[20,380] = 2.07, p = 0.0047; *AAV-Grn-treated Grn−/− mice vs AAV-GFP-treated Grn−/− mice, p = 0.001, Fisher’s post hoc test). AAV-Grn also reduced CatD activity in wild-type mice (†2-way RM-ANOVA of AAV-GFP-treated vs AAV-Grn-treated wild-type mice: effect of virus F[1,11] = 9.5, p = 0.0104, virus × time interaction F[20,220] = 6.432, p < 0.001). c, d, Consistent with their elevated CatD activity, Grn−/− mice exhibited elevated levels of pro-CatD (ANOVA effect of genotype F[1,31] = 8.758, p = 0.0059, **p = 0.01, Tukey’s post hoc test) and mature CatD (ANOVA effect of genotype, F[1,31] = 51.83, p < 0.0001, ***p < 0.001, Tukey’s post hoc test). AAV-Grn significantly reduced levels of pro-CatD in Grn−/− mice (ANOVA genotype × virus interaction F[1,31] = 7.102, p = 0.0121, *p < 0.05, Tukey’s post hoc test), although levels of mature CatD were not significantly reduced. In contrast, the reduced CatD activity of AAV-Grn-treated wild-type mice was associated with reduced levels of mature CatD (ANOVA effect of virus F[1,31] = 13.99, p = 0.0007, *p < 0.05, Tukey’s post hoc test), but not pro-CatD. Grn−/− mice also exhibited elevated levels of the lysosomal membrane protein LAMP-1 (ANOVA effect of genotype F[1,31] = 82.65, p < 0.0001, ****p < 0.0001, Tukey’s post hoc test) that were significantly reduced by AAV-Grn (ANOVA effect of virus F[1,31] = 22.41, p < 0.0001, virus × genotype interaction F[1,31] = 12.39, p = 0.0014). LAMP-1 levels were not significantly reduced by AAV-Grn in wild-type mice. e, f, Both mature CatD and pro-CatD protein levels were strongly correlated with CatD activity in AAV-Grn-treated wild-type mice (n = 6 AAV-Grn-treated wild-type mice, Pearson correlation pro-CatD: r = 0.9135, r² = 0.8345, p = 0.0109, mature CatD: r = 0.9259, r² = 0.8527, p = 0.008) (Figure legend continues.)
AAV-GFP-treated Grn<sup>−/−</sup> mice (Fig. 9b). AAV-Grn significantly reduced CatD activity in Grn<sup>−/−</sup> mice to levels similar to wild-type mice. AAV-Grn also reduced CatD activity in wild-type mice. We further explored these changes by immunoblotting for CatD.

Immunoblotting for CatD revealed potentially different mechanisms behind the reduction in CatD activity in Grn<sup>−/−</sup> and wild-type mice. As expected, Grn<sup>−/−</sup> mice treated with AAV-GFP exhibited increased levels of pro-CatD and mature CatD relative to wild-type (Fig. 9c,d). AAV-Grn reduced pro-CatD levels in Grn<sup>−/−</sup> mice to levels not significantly higher than AAV-GFP-treated wild-type mice (Fig. 9c,d). A similar pattern was observed with levels of mature CatD, although the effect of AAV-Grn did not reach significance in Grn<sup>−/−</sup> mice (Fig. 9c,d). In contrast, AAV-Grn strongly reduced levels of mature CatD (decreased by ~39%) in wild-type mice, whereas levels of pro-CatD did not differ from AAV-GFP-treated wild-type mice (Fig. 9c,d). To further test the association between CatD protein levels and activity in AAV-Grn-treated mice, we analyzed their correlation (Fig. 9e,f). We observed strong correlations between the fluorescence generated at the final time point of the CatD assay and both mature CatD and pro-CatD in AAV-Grn-treated wild-type and Grn<sup>−/−</sup> mice. The CatD immunoblotting data suggest that the reduction in CatD activity in Grn<sup>−/−</sup> mice is caused by a reduction in CatD expression without a change in CatD maturation, but in wild-type mice is caused by a reduction in CatD maturation without a change in CatD expression. This hypothesis was further supported by analysis of the ratio of mature/pro-CatD, which revealed a strong reduction in the mature/pro-CatD ratio in AAV-Grn-treated wild-type mice, but no significant change in AAV-Grn-treated Grn<sup>−/−</sup> mice (Fig. 9g).

We next analyzed the relative activity (fluorescence at 40 min time point divided by mature CatD protein levels) of CatD to better understand the effects of AAV-Grn on CatD activity. Despite a reduction in both raw activity and mature CatD levels, AAV-Grn-treated wild-type mice exhibited a ~50% increase in relative CatD activity (Fig. 9h), indicating that progranulin may enhance the per-molecule activity of CatD in wild-type mice. In contrast, AAV-Grn-treated Grn<sup>−/−</sup> mice did not exhibit a significant increase in relative CatD activity (Fig. 9h) despite the normalization of raw CatD activity (Fig. 9b) and partial normalization in protein levels (Fig. 9c,d). These data again demonstrate that AAV-Grn has different effects on CatD in wild-type and Grn<sup>−/−</sup> mice and show a partial, although incomplete, normalization of CatD abnormalities in Grn<sup>−/−</sup> mice.

To further understand the lysosomal changes in AAV-Grn-treated Grn<sup>−/−</sup> mice, we measured LAMP-1 levels in AAV-treated mice as an additional measure of lysosomal dyshomeostasis. As expected, AAV-GFP-treated Grn<sup>−/−</sup> mice exhibited increased LAMP-1 levels relative to AAV-GFP-treated wild-type mice (Fig. 9c,d). AAV-Grn significantly reduced LAMP-1 levels in Grn<sup>−/−</sup> mice (Fig. 9c,d). Unlike CatD, LAMP-1 levels were not significantly altered by AAV-Grn in wild-type mice. These data suggest that AAV-Grn causes a general reduction in the elevated levels of lysosomal proteins in the brain of Grn<sup>−/−</sup> mice, whereas its effects in wild-type mice may be more specific for CatD and other progranulin-interacting proteins.

**Discussion**

This study shows that AAV-mediated restoration of progranulin to Grn<sup>−/−</sup> mice after the onset of pathology improves lipofuscinosis and microgliosis across widespread brain regions, providing in vivo preclinical data supporting progranulin gene therapy or related progranulin replacement approaches. Several of our findings are informative for the future design of such therapies, including the beneficial effects observed with even low levels of progranulin and selective targeting of neurons, and the fact that sortilin binding is not required. AAV-expressed progranulin was delivered to the lysosome and improved lysosomal abnormalities, including normalizing CatD activity and partially normalizing CatD and LAMP-1 protein levels. The findings also shed light on progranulin biology and pathogenic mechanisms in progranulin deficiency. We observed improvement in microgliosis, although progranulin was delivered selectively to neurons, suggesting that the microglial response in progranulin deficiency occurs at least in part in response to neuronal dysfunction.

Our data suggest that even low levels of progranulin may be beneficial under conditions of progranulin deficiency. We observed widespread reduction of lipofuscinosis and microgliosis 8–10 weeks after AAV administration to Grn<sup>−/−</sup> mice (Figs. 3, 4) despite very modest effects of AAV-Grn on progranulin levels outside of the mPFC (Fig. 1). Progranulin protein levels in AAV-Grn-treated Grn<sup>−/−</sup> mice were not significantly increased outside of the mPFC when measured by ELISA, although immunostaining showed small numbers of progranulin-immunoreactive cells throughout the brain. The beneficial effects of such small increases in progranulin may be due to the requirement for nearly complete progranulin deficiency to develop NCL, as has been observed with other lysosomal storage disorders (Sandhoff and Harzer, 2013; Schulze and Sandhoff, 2014).

The apparent nonself reaction of Grn<sup>−/−</sup> mice to AAV-Grn was an unexpected result, but likely has very limited clinical significance. A nonself reaction to progranulin is unlikely in FTD-GRN patients, who still have 25–30% of normal circulating progranulin levels (Finch et al., 2009). In support of this idea, we did not observe mPFC inflammation in a recent study after identical injections of AAV-Grn in Grn<sup>−/−</sup> mice (Arrant et al., 2017). In addition, wild-type mice treated with AAV-Grn, unlike those treated with AAV-GFP, did not develop antibodies to the virally expressed protein, showing that wild-type mice did not recognize AAV-expressed progranulin as a foreign protein. For NCL patients with near-complete progranulin deficiency, the possibility of a nonself reaction may depend on the patient’s specific loss-of-function mutation (Smith et al., 2012; Almeida et al., 2016). Some GRN mutations do not cause complete loss of the protein, but allow generation of nonfunctional mutant progranulin proteins (Shankaran et al., 2008; Wang et al., 2010). Even low levels of endogenous progranulin protein could prevent a nonself reaction in patients, but the Grn<sup>−/−</sup> mouse line used in this study has deletion of the entire coding region of the Grn gene (Martens et al., 2012). This complete lack of progranulin may have led to progranulin’s recognition as a nonself protein and is likely spe-
cific to this type of experimental model, thus limiting the clinical significance of this result.

The nonself reaction of Grn−/− mice to AAV-Grn also appears to have had limited confounding effects on our study. The inflammation associated with the reaction was largely limited to the mPFC because microgliosis was reduced in regions outside of the mPFC (Fig. 4). The nonself reaction at the mPFC injection site did not appear to have adverse functional effects because mPFC-dependent behaviors were not impaired in AAV-Grn-treated Grn−/− mice (Fig. 5c,f). In addition, it is unlikely that the microgliosis induced by the nonself reaction mediated the improvement in lipofuscinosis due to increased microglial clearance of lipofuscin because microgliosis was decreased in the two brain regions where lipofuscinosis was significantly improved: CA3 and VPM/VPL thalamus (Figs. 3, 4). Therefore, the nonself reaction to AAV-Grn does not appear to confound the measures of lipofuscinosis and lysosomal dysfunction in regions distal to the injection site. However, the nonself reaction of Grn−/− mice to AAV-Grn likely explains the reduced expression of progranulin in AAV-Grn-treated Grn−/− mice versus wild-type mice (Fig. 1c,e). Prior studies have shown that immune responses to AAV transgenes can suppress expression of the AAV transgene, although therapeutic benefits may still be observed (Yuasa et al., 2007; Mendell et al., 2010; Haurigot et al., 2013). In two studies, nonself reactions to AAV transgenes provoked a cell-mediated immune response in which effector T cells killed muscle cells expressing the AAV transgene, resulting in gradual loss of transgene expression (Yuasa et al., 2007; Mendell et al., 2010). This may also occur in the brain because expression of nonself proteins in rats induces an immune response with high MHCII expression and T-cell infiltration at the injection site, production of antibodies against the AAV transgene, and loss of neurons and transgene expression at the injection site (Ciesielska et al., 2013). A similar mechanism could be at work in AAV-Grn-treated Grn−/− mice given the massive increase in MHCII expression at the AAV-Grn injection site. If so, then longer exposure of Grn−/− mice to AAV-Grn could result in detectable adverse outcomes such as neuronal loss and functional deficits. However, this is likely of limited clinical relevance because most Grn mutation carriers are heterozygous for loss-of-function mutations.

Our findings shed light on interactions between neurons and microglia, the primary cell types expressing progranulin in the brain. The improvement in microgliosis in regions distal to the mPFC in the absence of detectable microglial progranulin demonstrates that increasing neuronal progranulin is sufficient to modulate microglial function. Our progranulin construct was secreted normally by cultured cells (data not shown) and progranulin secreted by neurons could in theory be taken up by microglia via multiple pathways (Hu et al., 2010; Zhou et al., 2015). The fact that we detected no microglial progranulin in AAV-Grn-treated Grn−/− mice suggests that this uptake may not be efficient in microglia in vivo. Alternatively, progranulin taken up by microglia may be cleaved into granulins in the lysosome (Holler et al., 2017), but we would still expect to detect low levels of intracellular progranulin and the antibody we used for immunostaining has been reported to detect granulins by Western blot (Zhou et al., 2017c). The improvement in microgliosis could therefore be an indirect result of improved neuronal function.

In contrast, selective deletion of neuronal progranulin is not sufficient to reproduce the microglial pathology of Grn−/− mice. Selective knock-out of neuronal progranulin using CaMKII-Cre or Nestin-Cre does not produce microgliosis (Arrant et al., 2017; Petkau et al., 2017). Normally, most brain progranulin is produced by neurons because Nestin-Cre neuronal progranulin knock-out mice exhibit 50–60% reduction of brain progranulin protein, whereas depletion of microglial progranulin with Cx3CR1-CreERT2 produces ~30% reduction of brain progranulin (Arrant et al., 2017; Krabbe et al., 2017; Petkau et al., 2017). The remaining microglia-derived progranulin can thus prevent microgliosis and lipofuscinosis in neuronal progranulin knock-out mice, although it is insufficient to prevent social behavior deficits such as those seen in Grn−/− mice (Arrant et al., 2017). Neuronal–microglial crosstalk will be an important area for future investigation of progranulin biology.

The improvement in lipofuscinosis and microgliosis in Grn−/− mice likely reflects some clearance of preexisting pathology. At the age of AAV administration (10–12 months) Grn−/− mice exhibit lipofuscinosis and microgliosis throughout the brain (Ahmed et al., 2010; Wils et al., 2012; Filiano et al., 2013). Although these pathologies continue to progress with age (Ahmed et al., 2010; Wils et al., 2012), the rate of progression is insufficient to explain the differences that we observed between AAV-GFP- and AAV-Grn-treated Grn−/− mice. We observed reductions of 40–50% for both lipofuscinosis (Fig. 1) and CD68 immunostaining (Fig. 2) over 8–10 weeks. These changes greatly exceed the magnitude of increases expected in that time frame in untreated Grn−/− mice if AAV-Grn simply arrested pathology because it takes ~11 months for lipofuscinosis to double (from 6 months to 16–18 months) and ~10 months for microgliosis to increase by ~50% (from 12 months to 21–23 months) (Ahmed et al., 2010; Wils et al., 2012). AAV-GFP-treated Grn−/− mice had similar pathology as un.injected Grn−/− mice, so AAV-GFP did not artificially worsen the Grn−/− phenotype. It is therefore likely that the beneficial effects of AAV-Grn in Grn−/− mice reflect clearance of preexisting pathology.

The clearance of lipofuscin in Grn−/− mice is likely due to improved lysosomal function. Recent studies of lysosomal function in progranulin-insufficient models have consistently found abnormalities in CatD, with cell culture models revealing reduced CatD activity (Valdez et al., 2017; Ward et al., 2017) and Grn−/− mouse brain revealing an age-dependent increase in CatD activity and RNA and protein levels (Wils et al., 2012; Götzl et al., 2014; Beel et al., 2017; Zhou et al., 2017b). The age-dependent increase in CatD in mouse brain is similar to the increased levels of CatD in postmortem FTD-GRN brain (Götzl et al., 2014) and may be a compensatory response to underlying lysosomal dysfunction. Although we did not perform functional assays that would clarify the status of CatD activity in living mouse brain, the normalization of postmortem CatD activity, pro-CatD levels, and LAMP-1 levels indicate a shift toward more normal lysosomal homeostasis in AAV-Grn-treated Grn−/− mice.

The reduction of CatD maturation and total activity after AAV-Grn treatment in wild-type mice was an unexpected finding because several studies suggest that progranulin may enhance CatD activity (Beel et al., 2017; Valdez et al., 2017). However, our finding that AAV-Grn enhanced relative CatD activity (activity/mature CatD protein levels) in wild-type mice is consistent with these prior studies. One potential interpretation of these data is that the high levels of progranulin in AAV-Grn-treated wild-type mice enhance in vivo CatD activity, resulting in a homeostatic reduction in CatD maturation. The mechanism behind these effects remains unclear, but will be an interesting topic for future investigation.
Progranulin-boosting therapies are a rational approach to treating patients with GRN mutations, and this study provides important preclinical data relevant to several possible strategies. First, this study together with our previous study in Grn−/− mice show that an AAV gene therapy approach can improve pathology and behavioral abnormalities caused by progranulin deficiency (Arrant et al., 2017). AAV gene therapy was shown to be effective in several NCL models (Griffey et al., 2006; Passini et al., 2006; Cabrera-Salazar et al., 2007; Sondhi et al., 2007; Macauley et al., 2012; Hughes et al., 2014; Katz et al., 2015) and recent studies in nonhuman primates have raised the possibility of widespread cortical transection with a single thalamic AAV injection (Yazdan-Shahmorad et al., 2018). Second, this study shows that progranulin’s beneficial effects are sortilin-independent, which supports the strategy of blocking sortilin-dependent progranulin uptake to increase levels of extracellular progranulin (Hu et al., 2010; Lee et al., 2014). Progranulin’s neurotrophic effects were shown to be sortilin-independent in primary cultured neurons (Gass et al., 2012) and our study provides the first in vivo evidence that progranulin’s beneficial effects do not require sortilin.

Because of the clear loss-of-function mechanism, progranulin-deficient neurodegenerative diseases present an attractive opportunity for effective intervention. Our data support the concept of progranulin gene therapy or related progranulin-boosting approaches and inform the future design of such treatments.

References


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