Restoring neuronal progranulin reverses deficits in a mouse model of frontotemporal dementia

Andrew E. Arrant, Anthony J. Filiano, Daniel E. Unger, Allen H. Young and Erik D. Roberson

Loss-of-function mutations in progranulin (GRN), a secreted glycoprotein expressed by neurons and microglia, are a common autosomal dominant cause of frontotemporal dementia, a neurodegenerative disease commonly characterized by disrupted social and emotional behaviour. GRN mutations are thought to cause frontotemporal dementia through progranulin haploinsufficiency, therefore, boosting progranulin expression from the intact allele is a rational treatment strategy. However, this approach has not been tested in an animal model of frontotemporal dementia and it is unclear if boosting progranulin could correct pre-existing deficits. Here, we show that adeno-associated virus-driven expression of progranulin in the medial prefrontal cortex reverses social dominance deficits in Grn+/− mice, an animal model of frontotemporal dementia due to GRN mutations. Adeno-associated virus-progranulin also corrected lysosomal abnormalities in Grn+/− mice. The adeno-associated virus-progranulin vector only transduced neurons, suggesting that restoring neuronal progranulin is sufficient to correct deficits in Grn+/− mice. To further test the role of neuronal progranulin in the development of frontotemporal dementia-related deficits, we generated two neuronal progranulin-deficient mouse lines using CaMKII-Cre and Nestin-Cre. Measuring progranulin levels in these lines indicated that most brain progranulin is derived from neurons. Both neuronal progranulin-deficient lines developed social dominance deficits similar to those in global Grn+/− mice, showing that neuronal progranulin deficiency is sufficient to disrupt social behaviour. These data support the concept of progranulin-boosting therapies for frontotemporal dementia and highlight an important role for neuron-derived progranulin in maintaining normal social function.

Center for Neurodegeneration and Experimental Therapeutics, Alzheimer’s Disease Center, and Evelyn F. McKnight Brain Institute, Departments of Neurology and Neurobiology, University of Alabama at Birmingham, Birmingham, AL, USA

Correspondence to: Erik D. Roberson
1825 University Blvd., SHEL 1110
Birmingham, AL 35294, USA
E-mail: eroberson@uabmc.edu

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Abbreviations: AAV = adeno-associated virus; CaMKII = calcium/calmodulin-dependent protein kinase II; ELISA = enzyme-linked immunosorbent assay; FTD = frontotemporal dementia; mPFC = medial prefrontal cortex

Introduction

Progranulin is a secreted glycoprotein that is expressed by many cell types throughout the body (Bateman and Bennett, 1998). Progranulin has diverse actions that vary by cell type; it acts as a growth factor in many cell types, including neurons, and has anti-inflammatory effects in myeloid cells (Cenik et al., 2012; Kleinberger et al., 2013; Nguyen et al., 2013). Progranulin is implicated in the pathogenesis of a variety of diseases, including cancer, diabetes, rheumatoid arthritis, and Alzheimer's disease (Cenik et al., 2012; Matsubara et al., 2012; Nguyen et al., 2013;
Minami et al., 2014). However, progranulin is most strongly tied to frontotemporal dementia (FTD), one of the leading causes of dementia after Alzheimer’s disease (Ratnave et al., 2002; Harvey et al., 2003; Rosso et al., 2003; Borroni et al., 2010).

FTD includes multiple clinical syndromes, the most common of which is behavioural variant FTD, which is characterized by behavioural and personality changes including apathy, disinhibition, social withdrawal and compulsive behaviour (Mackenzie et al., 2010; Rasovsky et al., 2011; Karageorgiou and Miller, 2014). Mutations in the progranulin gene, GRN, are one of the most common genetic causes of FTD, causing as much as 5–10% of all FTD cases (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006). FTD-associated GRN mutations occur throughout the gene and disrupt function of the mutant allele through a variety of mechanisms, including nonsense-mediated decay and impairment of progranulin secretion (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006; Shankaran et al., 2008; Wang et al., 2010). As a result, GRN mutation carriers have >50% depletion of plasma progranulin levels (Finch et al., 2009).

In light of the human genetics, Grn+/− and Grn−/− mice provide animal models for understanding the effects of progranulin insufficiency. These mice develop behavioural deficits that may model aspects of FTD, particularly behavioural variant FTD. Both Grn+/− and Grn−/− mice develop social deficits and exhibit impaired fear memory, as seen in patients with behavioural variant FTD (Kayasuga et al., 2007; Hoefer et al., 2008; Yin et al., 2010b; Ghoshal et al., 2012; Filiano et al., 2013). After exposure to a novel, social environment, Grn+/− and Grn−/− mice have reduced activation of the central amygdala, a region that degenerates in FTD, which may indicate impaired local or circuit-level responses to social stimuli (Filiano et al., 2013). Taken together, work with Grn+/− and Grn−/− mice supports the hypothesis that progranulin insufficiency causes FTD in patients with GRN mutations.

The mechanism by which progranulin insufficiency may cause FTD is unknown, but experimental models indicate several potential mechanisms, including loss of progranulin’s neurotrophic effects, increased inflammation, and lysosomal dysfunction. In the brain, progranulin is primarily expressed by neurons and microglia (Ryan et al., 2009; Petkau et al., 2010). Progranulin has neurotrophic effects in cultured neurons, so neuronal progranulin insufficiency could impair neuronal function due to loss of neurotrophic support (Van Damme et al., 2008; Ryan et al., 2009; Chitramuthu et al., 2010; Gass et al., 2012; De Muynck et al., 2013). Progranulin also regulates inflammatory responses in microglia and macrophages, so microglial progranulin insufficiency could lead to inflammation (Yin et al., 2010a; Martens et al., 2012; Jackman et al., 2013). Microglia are responsible for synapse loss and neuronal dysfunction in Grn−/− mice, though it is unclear if this occurs in Grn+/− mice, which develop the same behavioural deficits as Grn−/− mice without clear signs of microgliosis (Filiano et al., 2013; Lui et al., 2016).

Finally, progranulin is critical for proper lysosomal function. Complete progranulin deficiency causes a lysosomal storage disorder, neuronal ceroid lipofuscinosis, and Grn−/− mice, but not Grn+/− mice, develop lipofuscin pathology (Smith et al., 2012; Gotzl et al., 2014). Progranulin insufficiency could therefore act through neurons, microglia, or both cell types to produce FTD.

The hypothesis that progranulin haploinsufficiency causes FTD raises the possibility that boosting progranulin levels in patients with GRN mutations will be an effective therapeutic strategy. There is support for this approach in cell culture models, in which restoring progranulin to progranulin-deficient neurons increases survival and enhances neurite outgrowth (Van Damme et al., 2008; Ryan et al., 2009; Gass et al., 2012). There are currently several approaches under development for boosting progranulin levels, including increasing expression from the intact allele, increasing extracellular progranulin by disrupting binding to its receptor sortilin, and gene therapy approaches (Hu et al., 2010; Capell et al., 2011; Cenik et al., 2011; Gass et al., 2012; Lee et al., 2014). However, progranulin-boosting approaches have not been tested in vivo in a model of FTD.

The goal of this study was to test in vivo the efficacy of boosting progranulin levels to correct abnormal behaviour and neuronal dysfunction in Grn+/− mice. In doing so, we aimed to address two therapeutically relevant questions. First, are progranulin-boosting therapies effective for treating existing deficits, or are they only effective if given before disease onset? Second, which cell type(s) should be targeted with progranulin-boosting therapies? To address these questions, we injected an adeno-associated virus (AAV) vector expressing mouse progranulin into the medial prefrontal cortex (mPFC) of 11–12-month-old Grn+/− mice. In follow-up experiments to further investigate the role of neuronal progranulin in the development of deficits in mice, we generated two neuron-specific progranulin knockout mouse lines by crossing CaMKII-Cre or Nestin-Cre-expressing mice with Grnflox/flox mice.

### Materials and methods

#### Animals

The Grn+/− mouse line used for the AAV study was generated and crossed onto a C57BL/6J background as previously described (Martens et al., 2012; Filiano et al., 2013). Neuronal progranulin-deficient (N-KO) mice were generated by crossing mice expressing CaMKII-Cre [Jackson laboratory Camk2a-cre T29-1 (Tsien et al., 1996)] or Nestin-Cre [Jackson laboratory Nestin-Cre 003771 (Tronche et al., 1999)] with Grnflox/flox mice (Martens et al., 2012). The CaMKII-Cre, Nestin-Cre, and Grnflox/flox lines used to generate N-KO mice had been previously bred onto a congenic C57BL/6J background. In Grn+/− and both N-KO lines, the entire
coding region of the progranulin gene was deleted in the null allele. Wild-type littermates were used as controls for Grn+/− mice, and Cre− littermates were used as controls for N-KO mice. Male and female mice were used for each experiment and data from each sex were combined after no sex-dependent differences were identified. The mice were maintained on a 12:12 h light/dark cycle with lights on at 06h00 and off at 18h00. All mice were housed in a barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The mice were given free access to laboratory rodent chow (Harlan, #7917) and water. Experiments were conducted during the light cycle (between 08h00 and 17h00) under ambient room lighting except where specified.

**Viral vectors and progranulin constructs**

The viral vector used to generate AAV-Grn was generated from a pcDNA4 vector containing the RNA-coding sequence of mouse progranulin provided by Joachim Herz. The RNA-coding sequence of mouse progranulin was amplified with a C-terminal myc tag using the following primers 5′ – AGCA GAGCTCTCTGGCTAAC – 3′ (forward) and 5′ – ATAG CGGCCGCGTTAATGCATATTCAGATCCTCTTCTGAG – 3′ (reverse). Mouse progranulin was then cloned into the CIGW AAV2 vector (rAAV2-CA-IRES-GFP-WPRE-rBG) (St Martin et al., 2007). Due to size limitations for efficient gene expression, the IRES-GFP was removed from the CIGW vector prior to cloning to generate the AAV-Grn construct (rAAV2-CA-mGrn-Myc-WPRE-rBG). The AAV-Grn construct was sent to the University of Pennsylvania Vector Core and packaged into an AAV1 capsid. An AAV-GFP vector with a matching AAV1 capsid was purchased from the UPenn Vector Core to serve as a control virus (cat. # AV-1-PV1963, AAV1-CB7-CI-eGFP-WPRE-rBG).

**Stereotaxic injection of AAV**

Prior to AAV injection, all mice were pretested in the tube test to determine the presence of social dominance deficits. Mice were anaesthetized with 5% isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments). Anaesthesia was maintained by administering isoflurane through a nose cone. An incision was made to expose the skull, and the coordinates of bregma and lambda were used to place skull into flat skull position. AAV-Grn or AAV-GFP were bilaterally injected into the mPFC at 1.9 mm anterior and 0.3 mm medial and lateral from bregma, and 2.2 mm below the surface of the skull. AAV (1 μl) was infused in each injection site at a titre of 7.36 × 1011 genomes/ml. After infusion, the injection holes were sealed with bone wax, the scalp incision was closed with surgical staples, and the mice were allowed to recover for 4–6 weeks before further testing.

**Tube test for social dominance**

The tube test protocol was conducted using our previously described protocol (Arrant et al., 2016). Mice of the same sex, but opposite genotype were placed in opposite ends of a 30.5 cm long tube and released simultaneously. The first mouse to place two paws outside of the tube was considered the loser. Each mouse was tested two to four times against different opponents to allow calculation of winning percentage for each mouse as well as overall wins per genotype. To prevent the mice from crossing over each other, while still having sufficient space to move through the tube, a 3.8 cm inner-diameter (I.D.) tube was used for male mice and a 3.2 cm I.D. tube was used for females. The tube was cleaned with 70% ethanol and dried between trials.

**Three-chamber sociability test**

The three-chamber sociability test was conducted under dim red lighting as previously described (Filiano et al., 2013). Mice were given a 10-min habituation session to the three chamber testing apparatus. After placement of a novel mouse in one chamber and a novel object in the other chamber, the mice were given a second 10-min session to investigate either the novel mouse or novel object. The time spent in each chamber and investigating the mouse or object was analysed using auto-mated video tracking software (Cleversys, Inc.).

**Novel, social environment**

Prior to the novel, social environment protocol, all mice were single-housed for 1–2 weeks. Mice of the opposite sex and same genotype were placed into a novel and social environment for 2 h as previously described (Scearce-Levie et al., 2008). After 2 h, mice were sacrificed and transcardially perfused with 0.9% saline. Brains were postfixed in 4% paraformaldehyde and stored in phosphate-buffered saline (PBS) until sectioning for immunostaining.

**Nesting behaviour**

Mice were single-housed in cages with a cotton nestlet (iso-BLOX, Harlan) for 24 h. The nestlet was weighed before and after the testing period, and the per cent of nestlet used was calculated as an index of nest building (Deacon, 2012).

**Conditioned fear training and extinction**

Conditioned fear training was conducted as previously described (Filiano et al., 2013). Mice were trained to associate an auditory cues (75 Db white noise) with an aversive foot shock (0.5 mA). The mice were placed in the testing chambers and given 180 s to acclimate prior to three cue/shock pairings with 40 s intertrial intervals. Each pairing consisted of a 20 s presentation of the auditory cue, with the foot shock given during the last 2 s of the cue. The mice were then returned to their home cages, and were subjected to extinction training 24 h later. Extinction training was conducted using a method adapted from Izquierdo et al. (2006). For extinction training, the testing chambers were altered by covering the foot shock grids with white plexiglass, with additional white plexiglass sheets used to alter the shape of the chamber. Mice were placed in the altered chambers for 120 s prior to 40, 30 s presentations of the auditory cue with 5 s between presentations. For analysis, the cue presentations were grouped into trial blocks with four presentations per block. Mice that did not
exhibit a doubling of freezing from baseline (Block 0) to Block 1 or that did not exhibit at least 20% freezing in Block 1 were considered to have insufficient fear memory and excluded from extinction analysis.

**Y-maze**

Y-maze testing was conducted as previously described (Li et al., 2014). The Y-maze consisted of three arms (38.1 cm long, 8.9 cm wide, 12.7 cm high) made of white plexiglass. Mice were placed in the hub of the maze and allowed to explore for 5 min. Activity was recorded and tracked with video tracking software (Cleversys). The order of entries into each arm was used to determine spontaneous alternation. A spontaneous alternation was defined as successive entries into all three arms without re-entry into a previous arm. The per cent spontaneous alternation was determined by dividing the number of spontaneous alternations by the number of total arm entries minus two (since a spontaneous alternation was not possible until the third entry).

**Progranulin ELISA**

Brain progranulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Adipogen) using the manufacturer’s instructions as previously described (Arrant et al., 2015). Brains were dissected and regions of interest were homogenized with lysis buffer (10 mM Tris, 10 mM NaCl, 3 mM MgCl2-6H2O, 1 mM EDTA, 0.05% NP-40). The samples were then diluted 1:1 with ELISA buffer (Adipogen) such that 30–50 μg of protein were loaded per well. AAV-GFP-treated mPFC samples were loaded at 1/10th the concentration of AAV-GFP-treated mPFC samples due to the high AAV-mediated expression of progranulin.

**Western blot**

Mouse prefrontal cortex samples were homogenized and fractionated to isolate synaptosomes (Hallett et al., 2008). LAMP1 western blots were performed on the S2 fraction (comprised of the cell lysate remaining after crude synaptosomes have been isolated). Western blot was then performed as previously described (Arrant et al., 2015). Blots were incubated overnight with a LAMP1 antibody (1:100, Developmental Studies Hybridoma Bank #1D4B), followed by an IRDye®-conjugated anti-rat secondary antibody (LI-COR Biotechnology). The blots were then probed with an α-tubulin antibody (1:1000, Sigma Aldrich #T5168) as a loading control. Blots were scanned with an Odyssey scanner (LI-COR Biotechnology). Western blots were performed on the S2 fraction (comprised of the cell lysate remaining after crude synaptosomes have been isolated). Western blot was then performed as previously described (Arrant et al., 2015). Blots were incubated overnight with a LAMP1 antibody (1:100, Developmental Studies Hybridoma Bank #1D4B), followed by an IRDye®-conjugated anti-rat secondary antibody (LI-COR Biotechnology). The blots were then probed with an α-tubulin antibody (1:1000, Sigma Aldrich #T5168) as a loading control. Blots were scanned with an Odyssey scanner (LI-COR Biotechnology) and quantitated with Image Studio Lite software (LI-COR Biotechnology).

**Immunostaining**

Immunohistochemistry was performed on 30 μm free-floating mouse brain sections as previously described (Palop et al., 2011). Primary antibodies were used at the following concentrations: CD68 (1:500; AbD Serotec #MCA1957), LAMP1 [1:250 for immunofluorescence, 1:2000 for diaminobenzidine (DAB)], Developmental Studies Hybridoma Bank #1D4B], myc-tag (1:1000 EMD Millipore #05-419, or 1:1000 Abcam #ab9106), c-fos (1:10000; EMD Millipore #PC38), NeuN (1:1000; EMD Millipore #MAB377), Iba1 (1:500; Wako #019-19741), GFAP (1:1000 for immunofluorescence, 1:5000 for DAB, Dako #Z0334), Progranulin (1:200 for immunofluorescence, 1:1000 for DAB; R&D Systems #AF2557). The sections were incubated in primary antibody overnight at 4°C prior to incubation in secondary antibodies for the appropriate species for 1 h at room temperature. Biotinylated secondary antibodies were used for colorimetric DAB immunostaining (Vector Laboratories), and Alexa Fluor®-conjugated secondary antibodies were used for immunofluorescent staining (Life Technologies). Fluorescent-labelled sections were counterstained with 1% Sudan Black (Acros Organics) to quench autofluorescence. For LAMP1 immunofluorescence, sections were counterstained with DAPI (Life Technologies) before mounting onto slides.

Immunostaining was imaged and analysed by several methods, depending on the application. Regional progranulin immunostaining was imaged by obtaining high-resolution scans with a slide scanner (PathScan Enabler IV, Meyer Instruments). Two measurements per mouse were conducted for each region. Regions of interest were then analysed by densitometry using ImageJ, with values from the corpus callosum and optic chiasm used to calculate background levels for adjacent brain regions.

C-Fos immunostaining was imaged at ×4 with an upright microscope (Nikon). A uniform threshold was applied to the images, which were then converted to binary and analysed with ImageJ’s particle counter function. CD68, GFAP, and LAMP1 colorimetric immunostaining, as well as autofluorescent lipofuscin deposits were analysed as previously described (Arrant et al., 2015). Two × 20 images per mouse were taken for each region of interest, and the pathology burden was quantified with ImageJ.

LAMP1 immunofluorescence was imaged at ×60 with a Leica TCS-SP5 laser scanning confocal microscope with 0.5 μm z-stacks taken through the entire depth of the section. For quantitation, a uniform threshold was applied to the image stacks and the percent of stack volume occupied by thresholded pixels was measured with ImageJ. Two stacks were taken per mouse, and their values were averaged. For representative images, LAMP1 immunostaining and DAPI staining were compressed into 2D maximum intensity projections.

Progranulin or myc co-labelling with NeuN, GFAP, or Iba1 was imaged at ×60 with a Leica TCS-SP5 laser scanning confocal microscope with 1 μm z-stacks taken through the entire depth of the section. In N-KO mice, the degree of progranulin co-labelling with NeuN or Iba1 was qualitatively scored using ImageJ. Maximum intensity projections were made of the progranulin channel (AF-488, green) and the NeuN or Iba1 channel (AF-594, red). These projections were converted to binary images using a uniform threshold to exclude background. Co-localization was then determined by overlaying the images using the ‘AND’ function of ImageJ’s image calculator. The resulting binary image containing all co-localized pixels was overlaid onto the original image of NeuN or Iba1 staining, and cells were qualitatively scored as having strong progranulin labelling throughout the cell, punctate progranulin labelling, or undetectable progranulin labelling by an observer blind to mouse genotype. Two images were counted for each cell type per mouse, and all counts were combined by genotype to give the overall distribution of cells with strong, punctate,
or undetectable co-localization. For representative images of all co-labelling studies, a noise reduction filter was applied in which each pixel was assigned the median value of a 3-pixel radius. The green and red channels were then scaled to a uniform brightness for all images and merged to provide the representative images.

## Quantitative RT-PCR

Grn mRNA levels were detected as previously described (Arrant et al., 2015). Brain RNA was isolated with TRIzol® reagent (Life Technologies). Grn mRNA was detected with a TaqMan® probe (Mm01245914_g1, Life Technologies) and normalized to β-actin (Actb, Mm00607939_s1, Life Technologies). Quantitative PCR was performed on a Roche LightCycler 480.

## Experimental design

To minimize the risk of false-positive findings due to excessive comparisons, we focused on tests in which we previously observed deficits in Grn+/− mice after a complete behavioural assessment (tube test, nesting, and novel, social environment) (Filiano et al., 2013). We also performed two prefrontal cortex-dependent tests (Y-maze and fear extinction) to confirm that AAV-Grn did not non-specifically alter behaviour. The number of animals needed for these studies were determined by power analysis based on the tube test, which has been the most robust behavioural phenotype of Grn+/− mice in our laboratory (Filiano et al., 2013; Arrant et al., 2016). Power analysis was conducted using a calculator freely available at http://www.stat.uiowa.edu/~rlenth/Power to achieve power of 0.8 to detect the expected losing phenotype of Grn+/− mice (Lenth, 2006–2009). A total of four independent cohorts of mice were run for the AAV experiment. For the neuronal progranulin knockout studies, we tested four independent cohorts of CaMKII-Cre N-KO mice and two independent cohorts of Nestin-Cre N-KO mice. For some measures such as c-Fos immunostaining, LAMP1 levels, or pathology, only a subset of mice were used, all of which came from the same cohorts. For the AAV experiment, animals were assigned to treatment groups after initial pretesting to assure that the AAV-GFP and AAV-Grn groups had similar behavioural phenotypes before AAV treatment. For all experiments, data collection and analysis were conducted with animals identified only by a unique number. Genotype and treatment information for each animal were not revealed until after data collection and analysis.

## Statistics

With the exception of tube test data and progranulin co-labeling with NeuN or Iba1, all data are presented as mean ± SEM. Two-tailed P-values were calculated for all analyses in the AAV study (Figs 1–3). One-tailed P-values were calculated for tube test data for neuronal progranulin knockout mice (Figs 6 and 8) and for post hoc analyses of progranulin ELISA and quantitative PCR data (Fig. 7), given the predicted directionality of changes. Two-tailed P-values were calculated for all other analyses with neuronal progranulin knockout mice. Normally-distributed data were analysed by ANOVA, repeated measures ANOVA, or t-test as described in the figure legends. Win totals from the tube test were analysed by the binomial test to compare observed versus expected distributions, with the expected win total set at 50% of the total matches for each genotype. Winning percentage from the tube test was analysed by Mann-Whitney test, but data are presented as distributions to allow clearer visualization of group differences. Raw winning percentages are shown in Supplementary Fig. 2. To plot the distribution of winning percentages, all values were binned into percentages of 0, 0.33, 0.66, or 1. Values falling between these bins were assigned to the next higher or lower bin in a counterebalanced fashion. Statistical analysis was performed prior to binning. The comparison of winning percentage of AAV-GFP-treated wild-type mice in their independent matches against AAV-GFP or AAV-Grn-treated Grn+/− mice was performed with a two-tailed Wilcoxon matched-pairs signed rank test. Nesting behaviour was analysed by Mann-Whitney test. All analyses other than extinction of conditioned fear were conducted with GraphPad Prism 7.0 (Graphpad) with α set at 0.05. Analysis of fear extinction was conducted with JMP Pro12 (SAS) with α set at 0.05.

## Study approval

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and adhered to guidelines set by the Office of Laboratory Animal Welfare and the American Association for Accreditation of Laboratory Animal Care.

## Results

### AAV-Grn reverses social dominance deficits in Grn+/− mice

To test the hypothesis that boosting progranulin levels could reverse deficits in Grn+/− mice, we expressed progranulin bilaterally in the mPFC of 11–12-month-old wild-type (Grn+/+) and Grn+/− mice using an AAV mouse progranulin vector (AAV-Grn) (Supplementary Fig. 1). We targeted the mPFC due to its importance in the social dominance behaviour, which is disrupted in Grn+/− mice (Wang et al., 2011; Arrant et al., 2016). Mice of both genotypes were injected with AAV-GFP to serve as controls. Prior to AAV injection, the mice were prescreened in the tube test for social dominance, which confirmed that the Grn+/− mice used for this study had the expected low social dominance phenotype (Fig. 1A) (Arrant et al., 2016).

Four to six weeks after AAV injection, we reassessed social dominance in the mice. Control AAV-GFP-treated Grn+/− mice still exhibited low social dominance versus wild-type mice, showing that AAV injections alone do not alter the low social dominance phenotype in Grn+/− mice (Fig. 1B). In contrast, AAV-Grn-treated Grn+/− mice no longer exhibited low social dominance versus AAV-GFP-treated wild-type mice (Fig. 1C), indicating a reversal of their prior social dominance deficit. In support of these
data, AAV-GFP-treated wild-type mice were significantly less dominant against AAV-Gm-treated Gm+/- mice than against AAV-GFP-treated Gm+/- mice (Fig. 1D). Furthermore, AAV-Gm-treated Gm+/- mice exhibited a strong winning phenotype versus AAV-GFP-treated Gm+/- mice (Fig. 1E). This within-genotype enhancement of social dominance did not occur in wild-type mice (Fig. 1F), indicating that AAV-Gm corrected underlying deficits in Gm+/- mice.
deficits in Grn+/– mice rather than non-specifically enhancing dominance.

**AAV-Grn enhances the amygdala response to a novel, social environment in Grn+/– mice**

We investigated whether AAV-Grn could improve deficits in the amygdala response to a novel, social environment in Grn+/– mice (Filiano et al., 2013). For this experiment, AAV-treated mice were placed in a novel environment with another mouse of the opposite sex, but same genotype for 2 h prior to sacrifice, after which their brains were immunostained for c-Fos to assess neuronal activation (Scearce-Levie et al., 2008). We previously observed reduced activation of the central amygdala (CeA) in Grn+/– mice with this protocol, indicating an impaired response of either the amygdala or a broader neural circuit to the novel, social environment (Filiano et al., 2013). Similar to the results with the tube test, AAV-Grn increased the number of c-Fos-positive cells in the central amygdala of Grn+/– mice, but not wild-type mice (Fig. 2). These data indicate that AAV-Grn corrects deficits in Grn+/– mice without non-specifically enhancing social behaviour and the brain response to novel, social stimuli.

**AAV-Grn does not alter other medial prefrontal cortex-dependent behaviours**

To further test whether the beneficial effects of AAV-Grn in Grn+/– mice reflected a correction of underlying deficits rather than a non-specific improvement in prefrontal cortical function, we tested the AAV-treated mice in two mPFC-dependent behaviour tests, Y-maze and conditioned fear extinction (Supplementary Fig. 3) (Yang et al., 2014; Giustino and Maren, 2015). We focused on mPFC-dependent behaviour because this was the site of AAV injection. We observed no effect of virus or genotype, or interaction of virus and genotype on these behaviours. These data show that AAV-mediated progranulin expression does not produce general improvements in behavioural performance, having effects only on Grn+/– mice in tests in which they show deficits. These data also indicate that virally mediated progranulin does not impair function of the region with the highest AAV-mediated progranulin expression, the mPFC.

**AAV-Grn normalizes levels of LAMP1 in the prefrontal cortex of Grn+/– mice**

Grn+/– mice develop severe lysosomal dysfunction, resulting in accumulation of lysosomal proteins in the brain (Gotzl et al., 2014). We hypothesized that Grn+/– mice might also develop lysosomal dysfunction, so we measured immunostaining for the lysosomal protein LAMP1 in prefrontal cortex of 12–13-month-old wild-type, Grn+/–, and Grn–/– mice. As expected, Grn–/– mice exhibited a strong increase in LAMP1 immunoreactivity (Fig. 3A), but Grn+/– mice exhibited a non-significant trend for increased LAMP1. To investigate Grn+/– mice with a more sensitive LAMP1 assay, we performed confocal microscopy for LAMP1 in the prefrontal cortex of wild-type and Grn+/– mice (Fig. 3B). With this approach, we found a significant elevation in LAMP1 immunoreactivity in Grn+/– mice. Similarly, we observed elevated LAMP1 levels in both
Grn\(^{+/+}\) and Grn\(^{−/−}\) mice by western blot of the prefrontal cortex (Fig. 3D). Taken together, these data support our hypothesis that Grn\(^{+/−}\) mice have lysosomal abnormalities, though to a milder extent than Grn\(^{−/−}\) mice. We then tested whether AAV-Grn could normalize LAMP1 levels in Grn\(^{+/−}\) mice. Control injections with AAV-GFP had no effect on the lysosomal abnormalities in Grn\(^{+/−}\) mice measured by either confocal microscopy (Fig. 3C) or western blot (Fig. 3E). In both cases, AAV-Grn-treated Grn\(^{+/−}\) mice had significantly lower LAMP1 levels than AAV-GFP-treated Grn\(^{+/−}\) mice, showing that AAV-Grn normalizes prefrontal cortical LAMP1 levels in Grn\(^{+/−}\) mice.

### AAV-Grn transduces neurons and increases progranulin throughout the forebrain

Given the beneficial effects of AAV-Grn in Grn\(^{+/−}\) mice, we next determined the magnitude, anatomic location, and cellular tropism of AAV-mediated progranulin expression to learn about where and by how much progranulin needs to be increased. To provide a qualitative screen for the spread of progranulin expression throughout the brain, we immunostained brains from AAV-treated mice for progranulin. As expected, AAV-Grn increased progranulin...
immunoreactivity strongly throughout the mPFC, and progranulin levels were high throughout medial brain structures, including the septum and medial areas of the striatum (Fig. 4A). Progranulin immunoreactivity was also increased in the medial regions of the thalamus, perhaps due to progranulin being released from mPFC axons (Petoukhov et al., 2013). To obtain a more sensitive and quantitative measure of progranulin expression in the mPFC and amygdala, progranulin ELISA was performed (Fig. 4B and C). Both wild-type and Grn+/− mice exhibited dramatic increases in progranulin levels in the mPFC (B, ANOVA main effect of virus, \( P < 0.0001 \)), with a similar level of expression in each genotype. AAV-Grn-treated mice also had a modest, but statistically significant increase in progranulin in the amygdala, restoring progranulin levels to around normal in Grn+/− mice (C, ANOVA main effect of virus, \( P = 0.0001 \)). In both regions, AAV-GFP-treated Grn+/− mice exhibited progranulin insufficiency as expected (B inset, C). Brains from AAV-treated mice were double-immunostained for the myc tag on virally-expressed progranulin and markers for neurons (D, NeuN), astrocytes (E, GFAP), or microglia (F, Iba1), revealing that of these three cell types, AAV-Grn only transduced neurons. Immunostaining and ELISA data were analysed by ANOVA. Tukey’s post hoc test was used for immunostaining and amygdala ELISA data. Genotype differences within viral group from the prefrontal cortex ELISA data were analysed by t-test due to the large difference in progranulin levels between AAV groups. Scale bars in representative double-labelled images represent 5 μm. \( n = 10–20 \) mice per group for progranulin immunostaining and 6–16 per group for progranulin ELISA. **\( P < 0.01 \), ***\( P < 0.001 \), ns = not significant.
mPFC and amygdala, two critical regions for FTD that we previously implicated in Grn+/− mice (Filiano et al., 2013; Arrant et al., 2016), we used a progranulin ELISA. The ELISA revealed very high expression of progranulin in the mPFC, around 35-fold above normal levels (Fig. 4B), and an ELISA revealed very high expression of progranulin in the mPFC, around 35-fold above normal levels (Fig. 4B), and a smaller, but significant increase in progranulin in the amygdala, where levels in AAV-Grn-treated Grn+/− mice were similar to Grn+/+ controls (Fig. 4C). Together, the immunostaining and ELISA data show that AAV-Grn drove progranulin expression throughout the forebrain, with highest expression at the injection site in the mPFC. AAV-GFP did not alter the expected progranulin deficiency in Grn+/− mice (Fig. 4B and C).

To address which cell type(s) were transduced by AAV-Grn, brain sections from AAV-Grn-treated mice were immunostained for the myc tag on AAV-derived progranulin and markers for neurons (NeuN), astrocytes (GFAP), or microglia (Iba1). These sections revealed strong neuronal progranulin-myc labelling (Fig. 4D) and an absence of astrocytic (GFAP) (Fig. 4E) or microglial progranulin-myc labelling (Fig. 4F). This result is consistent with previous studies of AAV1 vectors, which have been reported to transduce neurons, with minimal astrocytic and no microglial transduction (Burger et al., 2004). The transduction of neurons, but not astrocytes or microglia by AAV-Grn indicates that increased expression of neuronal progranulin is sufficient to improve social deficits in Grn+/− mice. However, AAV-mediated progranulin secreted from neurons (Tsien et al., 1996). To confirm the cell-type specificity of progranulin depletion in CaMKII-Cre N-KO mice, we performed double-immunostaining for progranulin and markers for neurons (NeuN) and microglia (Iba1) and qualitatively scored the images for co-localization of progranulin and markers for neurons in the frontal cortex and for microglia in the corpus callosum (Fig. 5). We observed a significant reduction in the degree of neuronal (Fig. 5A and B), but not microglial (Fig. 5C and D), progranulin immunolabelling, showing that CaMKII-Cre N-KO mice had specific depletion of neuronal progranulin.

We also generated a pan-neuronal progranulin knockout mouse line with Nestin-Cre (Tronche et al., 1999). Nestin-Cre is expressed during embryonic development in CNS precursor cells that give rise to neurons and glial cells such as astrocytes and radial glia (Zimmerman et al., 1994). However, these glial cell types do not appear to express progranulin in mice and likely contribute very little to the total pool of brain progranulin (Petkau et al., 2010). We therefore bred Nestin-Cre-expressing mice with Grnfl/fl mice to generate pan-neuronal progranulin knockout mice. As with CaMKII-Cre N-KO mice, Nestin-Cre N-KO mice had reduced progranulin immunolabelling in neurons (Fig. 5E and F), but not microglia (Fig. 5G and H).

**Neuronal progranulin deficiency is sufficient to disrupt social behaviour**

We first assessed the behavioural phenotypes of CaMKII-Cre N-KO mice. When tested at 18–22 months of age, CaMKII-Cre N-KO mice exhibited low social dominance in the tube test (Fig. 6A), similar to the phenotype observed in global Grn+/− mice. Mice expressing CaMKII-Cre without any floxed genes did not exhibit social dominance deficits at 16 months of age, indicating the deficits of CaMKII-Cre N-KO mice were due to loss of neuronal progranulin (Supplementary Fig. 4). In addition to their social dominance phenotype, CaMKII-Cre N-KO mice developed reduced sociability in the three-chamber sociability test (Supplementary Fig. 5), but did not develop a deficit in conditioned fear (Supplementary Fig. 5), which has been previously observed in global Grn+/− and Grn+/ mice (Filiano et al., 2013). CaMKII-Cre N-KO mice also developed a deficit in nesting behaviour, an ethologically relevant behaviour that is disrupted in other mouse models of neurodegeneration (Fig. 6B) (Filali et al., 2009; Deacon, 2012). These data show that neuronal progranulin depletion with CaMKII-Cre is sufficient to reproduce most of the social deficits observed in global Grn+/− mice.

We next tested whether neuronal progranulin depletion with CaMKII-Cre is sufficient to disrupt the amygdala response to a novel, social environment. We exposed Cre– and CaMKII-Cre N-KO mice to a novel, social environment prior to immunostaining brains for c-Fos. While global Grn+/− and Grn+/ mice have a specific reduction in central amygdala activation in this paradigm, CaMKII-Cre N-KO mice exhibited a reduction in activation across the amygdala (basolateral, central, and medial) (Fig. 6C and D).

**Neuronal progranulin depletion with CaMKII-Cre is not sufficient to cause gliosis and lipofuscinosis**

We next investigated whether neuronal progranulin deficiency is sufficient to cause pathology similar to that observed in global Grn+/− mice. Grn+/− mice develop gliosis and lipofuscinosis, with these phenotypes well-established by 12 months (Ahmed et al., 2010; Yin et al., 2010b; Wils et al., 2012; Filiano et al., 2013; Gotzl et al., 2014;
Figure 5  

CaMKII-Cre and Nestin-Cre selectively deplete neuronal progranulin. To confirm the expected neuron-specific progranulin depletion in CaMKII-Cre and Nestin-Cre N-KO mice, co-localization was assessed between progranulin and markers for neurons (NeuN, A and E) and microglia (Iba1, C and G). Co-localization was qualitatively scored between NeuN and progranulin in the frontal cortex, which showed a significant reduction in neuronal progranulin labelling with CaMKII-Cre (B, Chi-square, $P < 0.0001$). To avoid interference from neuronal progranulin, microglial progranulin labelling was scored in the corpus callosum (D, Fisher’s exact test, $P = 0.9999$), with no significant effect of CaMKII-Cre detected. Similarly, NeuN/progranulin co-localization was reduced in Nestin-Cre N-KO mice (F, Chi-square, $P < 0.0001$), while Iba1/progranulin co-localization was not changed (H, Fisher’s exact test, $P = 0.6461$). Representative ×60 double-labelled images are shown adjacent to the corresponding monochrome image of the green channel (progranulin), with 10 μm scale bars. For quantification of double-immunostaining, two images were counted per mouse, from four to six mice per genotype from each line. A total of 706–749 neurons and 91–97 microglia were counted per genotype in CaMKII-Cre N-KO mice. 356–609 neurons were counted for NeuN/progranulin co-labelling and 75–82 microglia were counted for Iba1/progranulin co-labelling in Nestin-Cre N-KO mice. Data were analysed by Chi-square test (for NeuN) and Fisher’s Exact test (for Iba1, used due to the presence of only two bins, as no microglia in either line had weak or absent progranulin immunolabelling). 

$***P < 0.0001$; ns = not significant.
Tanaka et al., 2014). To test for these phenotypes in CaMKII-Cre N-KO mice, we measured markers of gliosis (GFAP, CD68), and autofluorescent lipofuscin granules in the frontal cortex, hippocampus, and thalamus of 20-month-old Cre– and CaMKII-Cre N-KO mice (Fig. 6E–G). Samples from Grn−/− mice were run in parallel as positive controls. We detected no differences between CaMKII-Cre N-KO mice and Cre– controls in any
measure of pathology, showing that neuronal progranulin depletion with CaMKII-Cre is not sufficient to reproduce the pathology of global Grn−/− mice.

The lack of detectable pathology in CaMKII-Cre N-KO mice could indicate that progranulin secreted from microglia is sufficient to prevent lipofuscin accumulation in progranulin-deficient neurons. This raised the interesting question about how much brain progranulin is produced by neurons versus microglia, and our progranulin N-KO lines provided an opportunity to answer this question in vivo.

Most brain progranulin is produced by neurons

Compared to controls, Grn RNA was ~50% lower in CaMKII-Cre N-KO mice in both frontal cortex and amygdala (Fig. 7A). Grn RNA levels were unchanged in thalamus where CaMKII-Cre is not expressed. Similarly, progranulin protein levels in CaMKII-Cre N-KO mice were about one-third lower than Cre− controls in frontal cortex and amygdala, but unchanged in thalamus (Fig. 7B).

We analysed brain progranulin protein levels with finer anatomic resolution using a semiquantitative densitometric approach on brains immunostained for progranulin (Fig. 7C and D). These data supported the ELISA and quantitative PCR data, showing significant depletion of progranulin in areas expected to express CaMKII-Cre. In the amygdala, progranulin was depleted in the basolateral amygdala, but not the central or medial amygdala. The hippocampus also showed subregional specificity, with significant progranulin depletion in CA1 and CA3, but not the dentate gyrus. Progranulin levels did not differ from Cre− controls in the striatum, hypothalamus, or thalamus of N-KO mice. These data indicate that a substantial fraction of brain progranulin is derived from neurons, but as CaMKII-Cre is expressed in only a subset of neurons (primarily excitatory principal cells), data from this line will underestimate the actual neuronal contribution to brain progranulin levels.

To better address this question, we examined the Nestin-Cre N-KO mice. As expected, Nestin-Cre produced a larger depletion of brain progranulin than CaMKII-Cre and affected nearly all brain regions measured. Pan-neuronal progranulin depletion with Nestin-Cre dramatically reduced brain progranulin, with Grn RNA levels reduced by 70–80% in the cortex, amygdala, and thalamus (Fig. 7E), and progranulin protein levels reduced by 50–60% in these same regions (Fig. 7F). Nestin-Cre N-KO mice had significant reduction of progranulin throughout the forebrain relative to Cre− controls, with reduced progranulin immunoreactivity in the cortex, hippocampus, amygdala, striatum, thalamus, and hypothalamus (Fig. 7G and H). These data show that under baseline conditions most brain progranulin is produced by neurons.

Nestin-Cre N-KO mice develop social deficits

Next, we examined behavioural phenotypes in these mice. Like the CaMKII-Cre N-KO mice, Nestin-Cre N-KO developed a losing phenotype in the tube test for social dominance at 16 months of age (Fig. 8A). Mice expressing Nestin-Cre without floxed genes did not develop a social dominance phenotype at 16 months of age (Supplementary Fig. 4), demonstrating that this effect is due to neuronal progranulin deficiency, not a non-specific effect of Cre expression. Nestin-Cre N-KO mice also had a trend for a deficit in nesting behaviour at 18–24 months of age (Fig. 8B) and exhibited impaired activation of the central and medial amygdala, but not the basolateral amygdala, after exposure to a novel environment at 18 months of age (Fig. 8C).

Nestin-Cre N-KO mice develop cortical astrogliosis, but lack any other aspects of global Grn−/− pathology

We assessed gliosis and lipofuscinosis in 18–19-month-old Nestin-Cre N-KO mice as previously discussed for CaMKII-Cre N-KO mice. Similar to CaMKII-Cre N-KO mice, Nestin-Cre N-KO mice did not differ from Cre− littermates in CD68 immunoreactivity (Fig. 8E) or lipofuscin accumulation (Fig. 8G) in the cortex, hippocampus (CA3), or thalamus (ventroposteromedial/lateral nuclei). However, Nestin-Cre N-KO mice had elevated GFAP immunoreactivity in the cortex of a similar magnitude as global Grn−/− mice (Fig. 8F). These data show that pan-neuronal progranulin depletion with Nestin-Cre is insufficient to replicate most of the pathology of Grn−/− mice, though it is sufficient to induce cortical astrogliosis.

Discussion

This study shows that boosting neuronal progranulin levels is sufficient to correct, and depleting neuronal progranulin is sufficient to create, behavioural deficits in mouse models of FTD. This indicates that neuronal progranulin levels are an important target for treatment of FTD due to GRN mutations, as well as an important factor in FTD pathogenesis. These data provide the first in vivo support for the use of progranulin-boosting therapies for FTD due to GRN mutations by demonstrating that restoring neuronal progranulin is sufficient to reverse abnormalities in social dominance and boost the amygdala response to a novel, social environment in Grn+/− mice. This study also provides evidence of subtle lysosomal abnormalities in Grn+/− mice that are corrected by expression of neuronal progranulin. These findings support the hypothesis that lysosomal dysfunction may play a role in FTD due to GRN mutations.
Figure 7 Neuronal progranulin depletion with CaMKII-Cre and Nestin-Cre significantly reduces total brain progranulin. CaMKII-Cre N-KO mice exhibited significant reductions in progranulin RNA in the frontal cortex and amygdala (A, ANOVA effect of genotype, \( P = 0.0096 \)). Progranulin protein levels were also reduced in the frontal cortex (FC) and amygdala (AMG) of CaMKII-Cre N-KO mice (B, ANOVA effect of genotype, \( P = 0.0189 \)). Grn RNA and protein levels were not reduced in the thalamus of CaMKII-Cre N-KO mice, which is consistent with low CaMKII-Cre expression in this brain region. Progranulin immunostaining (C and D) was used to provide a semiquantitative screen of progranulin levels across the brain, and revealed progranulin depletion in the cortex (cingulate and insula), hippocampus (CA1 and CA3), and basolateral amygdala of CaMKII-Cre N-KO mice (repeated measures ANOVA effect of genotype \( P = 0.0039 \), genotype \( \times \) region interaction \( P < 0.0001 \)). Nestin-Cre N-KO mice exhibited even stronger reductions in total progranulin levels throughout the brain. Total progranulin RNA (E, repeated measures ANOVA effect of genotype, \( P < 0.0001 \)) and protein (F, repeated measures ANOVA effect of genotype, \( P < 0.0001 \)) were reduced in frontal cortex (FC), amygdala (AMG), and thalamus (Thal). Analysis of progranulin immunoreactivity (G) showed strong reduction of
The reversibility of deficits in Grn+/- mice is a key finding of this study, and supports the use of progranulin-boosting therapies in FTD patients with GRN mutations. Progranulin-boosting therapies are an exciting approach for treatment of FTD due to GRN mutations, and these data provide vital in vitro support for this approach. Our findings are consistent with prior studies showing that deficits in progranulin-deficient cultured cells may be reversed by boosting progranulin levels (van Damme et al., 2008; Gass et al., 2012). Of course, it must be noted that Grn+/- mice likely model early-stage FTD, as they exhibit social and emotional behaviour deficits without TDP-43 pathology, neuronal loss, gliosis, or atrophy (Ahmed et al., 2010; Filiano et al., 2013), so this study is likely to be most relevant to early-stage FTD cases.

The second key finding of this study is the critical role of neuron-derived progranulin in the development of social dominance and amygdala activation deficits in mice, which in turn may indicate a critical role for neuron-derived progranulin in FTD due to GRN mutations. One line of supporting evidence derives from the AAV experiments. We generated a vector with an AAV1 capsid, which transduces neurons with very poor transduction of glial cells (Burger et al., 2004). We confirmed that this AAV-Grn vector drove exogenous progranulin expression in neurons but not microglia or astrocytes, indicating that neuronal progranulin restoration is sufficient to correct deficits in Grn+/- mice (Fig. 4). Another line of evidence comes from the selective depletion of neuronal progranulin in both N-KO mouse lines, which demonstrates the necessity of neuron-derived progranulin to prevent many of the deficits seen in Grn+/- mice.

Our finding that total brain progranulin was significantly depleted in N-KO mice further supports neuronal progranulin as an important factor in FTD due to GRN mutations. While neurons and microglia have been considered the two primary sources of brain progranulin, the relative contribution of each cell type has been unclear (Daniel et al., 2000; Petkau et al., 2010). Cultured microglia have much higher progranulin expression than cultured neurons, and a study of microglia and neurons isolated by fluorescence-activated cell sorting produced similar results (Martens et al., 2012; Zhang et al., 2014). However, microglial progranulin expression is strongly increased when microglia are activated by injury or inflammatory stimuli, raising the possibility that microglial progranulin expression is upregulated by culturing or cell sorting (Moisse et al., 2009; Naphade et al., 2010; Petkau et al., 2010; Tanaka et al., 2013). The advantage of the selective knockout approach to addressing this question is that it provides a measure of brain progranulin without requiring manipulations that activate microglia. Our ELISA and quantitative PCR data from Nestin-Cre N-KO mice show that in the absence of injury or inflammation, more than 50% of brain progranulin expression is derived from neurons (Fig. 7). We consider the Nestin-Cre data to provide the most accurate assessment of neuronal contribution to total brain progranulin, as CaMKII-Cre does not target inhibitory interneurons (Tsien et al., 1996) and may not express in every excitatory neuron.

An important caveat to consider in interpreting both the AAV and N-KO experiments is that progranulin is a secreted protein, so changes in neuronal progranulin likely affect other cells in the brain, including microglia. In the AAV experiment, virally-delivered progranulin secreted by transduced neurons could be taken up by neighbouring microglia. This might not be detected by myc tag immunostaining or commercially available progranulin antibodies, as progranulin can be trafficked to the lysosome and cleaved into granulin fragments (Cenik et al., 2012). Similarly, under normal conditions, neuronally secreted progranulin could regulate nearby microglia, so in the N-KO mouse experiments, reduction of neuronal progranulin could have effects on microglia (although not leading to measurable microgliosis) (Figs 6, 8 and Supplementary Fig. 5). Therefore, our data do not rule out a role for microglia in the pathogenesis of progranulin-insufficient FTD. The data do, however, strongly support a critical role for neurons in this disease and highlight the importance of neuronal-derived progranulin in preventing social deficits.

In addition to potential effects of neuron-secreted progranulin on microglia, we considered the potential effects of microglia-secreted progranulin on neurons. An effect of microglial progranulin on neurons is the most likely explanation for the lack of lipofuscinosis in both N-KO lines, despite the complete deletion of progranulin from neurons expressing Cre (Figs 6 and 8). We hypothesize that these progranulin-deficient neurons took up low levels of extracellular progranulin that was secreted by microglia, preventing lipofuscin accumulation that is seen in Grn+/+ mice. We did not detect direct evidence of neuronal uptake, as most neurons in both N-KO lines, especially the Nestin-Cre line (Fig. 5E and F), had...
undetectable progranulin levels. Of course, it is possible that these neurons did take up microglial progranulin that was subsequently cleaved into granulins that our antibodies cannot detect. However, it is important to emphasize that even if the neurons in N-KO mice take up some microglial progranulin, the fact that these mice developed behavioural deficits similar to those in global Grn−/− mice indicates that uptake of progranulin from microglia is insufficient to replace a critical function of neuron-derived progranulin.

This observation that normal microglial progranulin cannot substitute for neuronal progranulin raises the

Figure 8 Pan-neuronal progranulin deficiency with Nestin-Cre impairs social dominance, nesting, and the amygdala response to a novel, social environment, and produces cortical astrogliosis. Nestin-Cre N-KO mice developed a losing phenotype in the tube test for social dominance (A, \(P = 0.0007\)), and a trend for reduced nesting behaviour (B, \(P = 0.057\)). Nestin-Cre N-KO mice also exhibited impaired activation of the amygdala after exposure to a novel, social environment (C, repeated measures ANOVA effect of genotype, \(P = 0.0092\)), with specific impairments in the central (\(P = 0.0167\)) and medial (\(P = 0.0225\)) amygdala. Representative images of c-Fos immunostaining are shown with lines drawn to illustrate boundaries between amygdala nuclei (D). The boxed areas in the central amygdala are enlarged in the panels below the top images. The scale bars in the larger image in D = 100 μm, and the scale bar in the smaller central amygdala image = 50 μm. For assessment of pathology, brains from 18–19 month-old Nestin-Cre N-KO mice and Cre− littermates were immunostained for markers of microgliosis (E, CD68) and astrogliosis (F, GFAP). The presence of autofluorescent lipofuscin granules was measured by imaging brain sections with an epifluorescence microscope (G). No differences were detected between in CD68 immunoreactivity (E) or lipofuscin accumulation (G) showing that neuronal progranulin deficiency is not sufficient to reproduce most of the pathology of Grn−/− mice. However, Nestin-Cre N-KO mice exhibited cortical astrogliosis (F, repeated measures ANOVA genotype × brain region interaction \(P = 0.0012\)). A limited number of brains from Grn−/− mice were processed in parallel with the sections to serve as a positive control, but were not included in the statistical analysis given the small sample size. Representative × 20 images of CD68, GFAP, and lipofuscin are shown for each group. Scale bars = 50 μm. Tube test and nesting data were analysed by Mann-Whitney test, and c-Fos and pathology data were analysed by repeated measures ANOVA with Sidak’s post hoc test. \(n = 23–24\) mice per genotype for the tube test, 24 per genotype for nesting, 16 per genotype for c-Fos, and 9–14 per genotype for pathology. BLA = basolateral amygdala; CeA = central amygdala; MeA = medial amygdala; Ctx = cortex; HPC = hippocampus; Thal = thalamus. \(*P < 0.05\), \(**P < 0.01\).
question of whether neuronally-expressed progranulin acts in an autocrine, or even intracrine, manner that may not be mimicked by extracellularly-derived progranulin. The possibility that progranulin may act in such an autocrine manner will be an important area for future study that could have therapeutic implications. For instance, FTD patients with GRN mutations exhibit elevated progranulin RNA levels in degenerated areas of the brain post-mortem, which is likely due to microglial activation and subsequent increased expression of microglial progranulin from the intact allele (Chen-Plotkin et al., 2010). As a result, FTD patients with GRN mutations did not have significantly reduced progranulin protein levels in degenerated brain regions, despite significant reduction in spared brain regions (Chen-Plotkin et al., 2010). As this elevated microglial progranulin does not appear to arrest disease progression, an important area for future study will be determining if specifically boosting neuronal progranulin levels is necessary for effective treatment of FTD in patients with GRN mutations.

In summary, this study provides critical in vivo data to support the development of progranulin-boosting therapies for FTD due to GRN mutations. In light of our findings, progranulin-boosting therapies represent a promising approach to developing the first true disease-modifying treatment for a neurodegenerative disease. In contrast to mutations in other FTD-related genes such as MAPT and C9orf72, where debate continues about whether disease is mediated through gain- or loss-of-function effects, GRN mutations are widely agreed to cause FTD through loss-of-function (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006). Thus, boosting progranulin levels is a rational approach to treating FTD due to GRN mutations, and several approaches are under development that could increase progranulin expression from the intact allele (Capell et al., 2011; Cenik et al., 2011; Lee et al., 2014; Holler et al., 2016). Given progranulin’s trophic and anti-inflammatory effects, progranulin boosting therapies could potentially be useful for treating a variety of other disorders of the nervous system. Methylation of the GRN gene, which is associated with lower expression levels, is elevated in sporadic FTD patients, perhaps indicating a role for progranulin and progranulin-boosting therapies in sporadic FTD (Banzhaf-Strathmann et al., 2013; Galimberti et al., 2013). GRN mutations are also associated with Alzheimer’s disease, and progranulin deficiency worsens amyloid pathology and behavioural outcomes in human amyloid precursor protein (hAPP) overexpressing mice (Brouwers et al., 2008; Lee et al., 2011; Perry et al., 2013; Minami et al., 2014). Progranulin overexpression improves amyloid pathology in progranulin-deficient, hAPP overexpressing mice, indicating a potential role for progranulin-boosting therapies in Alzheimer’s disease (Minami et al., 2014). Additionally, viral progranulin overexpression protects tyrosine hydroxylase-positive neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson’s disease (Van Kampen et al., 2014). Finally, progranulin-deficient mice exhibit worse outcomes than wild-type mice in various models of injury to the nervous system, including traumatic brain injury, ischaemia, and inflammatory insults (Martens et al., 2012; Jackman et al., 2013; Tanaka et al., 2013). Progranulin-boosting therapies thus have strong potential to treat FTD due to GRN mutations, but may also be useful for a wide variety of other diseases and injuries to the central nervous system. This study provides critical in vivo evidence for the efficacy of such progranulin-boosting therapies.

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Supplementary material

Supplementary material is available at Brain online.

References

Deacon R. Assessing burrowing, nest construction, and hoarding in brain 2017: 140; 1447–1465 A. E. Arrant
Giustino TF, Maren S. The role of the medial prefrontal cortex in the conditioning and extinction of fear. Front Behav Neurosci 2015; 9: 298.
Hallett PJ, Collins TL, Standaert DG, Dunah AW. Biochemical fractionation of brain tissue for studies of receptor distribution and trafficking. Curr Protoc Neurosci 2008 Chapter I: Unit 1.16


