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Conditional loss of progranulin in neurons is not sufficient to cause neuronal ceroid lipofuscinosis-like neuropathology in mice

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ABSTRACT

Progranulin deficiency due to heterozygous null mutations in the *GRN* gene is a common cause of familial frontotemporal lobar degeneration (FTLD), while homozygous loss-of-function *GRN* mutations cause neuronal ceroid lipofuscinosis (NCL). Aged progranulin-knockout mice display highly exaggerated lipofuscinosis, microgliosis, and astrogliosis, as well as mild cell loss in specific brain regions. Progranulin is a secreted glycoprotein expressed in both neurons and microglia, but not astrocytes, in the brain. We generated conditional progranulin-knockout mice that lack progranulin in nestin-expressing cells (Nes-cKO mice), which include most neurons as well as astrocytes. We confirmed near complete knockout of progranulin in neurons in Nes-cKO mice, while microglial progranulin levels remained similar to that of wild-type animals. Overall brain progranulin levels were reduced by about 50% in Nes-cKO, and no Grn was detected in primary Nes-cKO neurons. Nes-cKO mice aged to 12 months did not display any increase in lipofuscin deposition, microgliosis, or astrogliosis in the four brain regions examined, though increases were observed for most of these measures in Grn-null animals. We conclude that neuron-specific loss of progranulin is not sufficient to cause similar neuropathological changes to those seen in constitutive Grn-null animals. Our results suggest that increased lipofuscinosis and gliosis in Grn-null animals are not caused by intrinsic progranulin deficiency in neurons, and that microglia-derived progranulin may be sufficient to maintain neuronal health and homeostasis in the brain.

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1. Introduction

Progranulin is a pleiotropic, secreted growth factor with widespread expression throughout the periphery and in the brain (Petkau and Leavitt, 2014). Within the central nervous system, progranulin is a putative lysosome-associated protein (Tanaka et al., 2014; Tanaka et al., 2017) expressed in most neuronal populations and in microglia (Baker et al., 2006; Chen-Plotkin et al., 2010; Petkau et al., 2010), and reduced expression of progranulin is associated with multiple neurodegenerative diseases (Petkau and Leavitt, 2014). Most notably, heterozygous loss-of-function mutations in the progranulin gene (*GRN*) are a common cause of familial frontotemporal lobar degeneration (FTLD) (Baker et al., 2006; Cruts et al., 2006), while homozygous loss of *GRN* expression causes neuronal ceroid lipofuscinosis (NCL) (Smith et al., 2012).

Progranulin expression, both in humans and in mice, is present in a punctate, perinuclear pattern in neurons throughout the brain, with highest expression occurring in the thalamus and CA3 layer of the hippocampus, and without obvious changes in expression in response to injury (Petkau et al., 2010). In microglia, expression of progranulin is notably stronger than in neurons, and is further increased when microglia are activated after stress or injury (Chen-Plotkin et al., 2010; Naphade et al., 2010; Petkau et al., 2010).

Mice homozygous for a targeted deletion of the mouse progranulin gene (*Grn*) develop a robust neuropathological phenotype with age (Ahmed et al., 2010; Ghoshal et al., 2012; Petkau et al., 2012; Wils et al., 2012; Yin et al., 2010b), though heterozygous Grn-targeted mice do not develop this phenotype (Ahmed et al., 2010; Petkau et al., 2012). Aged Grn-null mice display exaggerated deposition of the aging pigment lipofuscin and accumulation of a NCL-like storage material, as well as robust astrogliosis and microgliosis in multiple brain regions (Ahmed et al., 2010; Ghoshal et al., 2012; Petkau et al., 2016; Wils et al., 2012; Yin et al., 2010b). In addition, mild neuronal cell loss (Ghoshal et al., 2012) and alterations in neuronal morphology have also been reported (Petkau et al., 2016; Petkau et al., 2012). Grn-null immune cells, such as macrophages and microglia, show a hyper-

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inflammatory response to various stimuli (Martens et al., 2012; Suh et al., 2012; Yin et al., 2010a), as well as altered lysosomal biogenesis (Tanaka et al., 2013a; Tanaka et al., 2013b; Tanaka et al., 2017). Grn deficiency in neurons has been associated with reduced neuronal survival (De Muynck et al., 2013; Guo et al., 2010; Kleinberger et al., 2010; Ryan et al., 2009; Van Damme et al., 2008), reduced neurite outgrowth (Van Damme et al., 2008), and altered lysosome function (Tanaka et al., 2017).

In this study, we sought to determine the relative contribution of progranulin derived from neurons to the robust neuropathological phenotype present in constitutive Grn-null mice. We created neuron-specific conditional Grn knockout mice (Nes-cKO) and evaluated whether they developed lipofuscinosis, microgliosis, and astrogliosis in the brain with age. Surprisingly, we find that near complete loss of Grn expression in most neurons is not sufficient to reproduce any of the neuropathological phenotypes observed in constitutive Grn-null mice, and conclude that microglia-derived Grn is sufficient to maintain brain homeostasis in terms of the outcomes measured here.

2. Methods

2.1. Mice

The generation of 'floxed' progranulin-targeted (Floxed) mice was previously described (Petkau et al., 2013). Conditional nestin-knockout (Nes-cKO) mice were generated by crossing homozygous $Grn^{flox/flox}$ mice on the C57Bl/6 background to transgenic mice expressing Cre recombinase under the control of the rat nestin promoter and enhancer (generous gift from Dr. E. Simpson; originally from The Jackson Laboratory, strain name: B6·Cg-Tg(Nes-cre)1Kln/J). Final experimental cohorts were generated by crossing $Grn^{flox/flox}$, Cre + animals to $Grn^{flox/flox}$, Cre- littermates. Genotyping was performed on tail tip DNA at wean and confirmed on a second DNA sample at sacrifice using the following primer sequences: Grn common forward primer: 5′- CGGAACA CAGTGTCCAGATG-3′; Grn intron 2 reverse primer: 5′- ATCAACCA AAGGGTCTGTGC-3′; Grn exon 5 reverse primer: 5′-GTGGCAGAGTCA GGACATTCAAACT-3′; Cre forward primer: 5′- GCGGTCTGGCAGT AAAAACTATC-3′; Cre reverse primer: 5′- GTGAAACAGCATTG CTGTCACTT-3′. To generate Het-Nes-cKO mice, we crossed $Grn^{flox/flox}$, Cre + mice to constitutive $Grn^{-/-}$ mice and genotyped the offspring for the presence of the Cre transgene.

Mice were housed on ventilated racks in specific pathogen-free barrier facility with a 12 h light/dark cycle. Mice were group-housed with their littermates to a maximum of four mice per cage. All animal procedures were done with the approval of the Canadian Council for Animal Care and the University of British Columbia's Animal Care Committee.

2.2. Protein extraction and quantification of Grn by ELISA

Whole brain lysate was prepared by homogenizing previously snap-frozen brains in a rotor-stator homogenizer for 30 s. in 1 mL of complete lysis buffer (50 mM Tris-HCl, 1% Triton-X, 150 mM NaCl, Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific), Halt protease inhibitor cocktail (Thermo Fisher Scientific)). Primary cortical neurons were generated from embryonic day 17.5 pups as previously described. Neurons cultured for 7 days in 6-well plates were washed twice with ice-cold PBS before being scraped into 60 μL of complete RIPA buffer (50 mM Tris-HCl, 1% Triton-X, 150 mM NaCl, 1% deoxycholic acid, 1 mM EDTA, Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific), Halt protease inhibitor cocktail (Thermo Fisher Scientific)) with DNase added. The lysate was collected into pre-chilled microcentrifuge tubes and stored at −80 °C until use. Total protein was assayed using Bradford reagent (BioRad). Primary microglia cultures were generated as previously described (Connolly et al., 2016). Conditioned media was collected after 24 h and stored at −20 °C until used.

The quantity of Grn in whole brain lysate, cell lysate, or conditioned media was determined by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Mouse progranulin ELISA; Adipogen, Korea). Microglia supernatant samples were diluted 1:5; for neuronal cell lysates, 20 μg of total protein was loaded per well, and for whole brain lysate, 100 μg of protein was used. All samples were run in duplicate. The ELISA was conducted according to the manufacturer's instructions. Data represent the average of 2–8 samples per condition, and all conditions that were compared directly were run on the same plate.

2.3. RNA isolation and qPCR

For analysis of *Grn* mRNA, cortical tissue from 4 Floxed, 5 Nes-cKO, and 2 GrnKO mice at 4–6 months of age was collected and immediately frozen at −80 °C. Samples were homogenized with a bead homogenizer in lysis buffer followed by total RNA extraction (PureLink RNA mini kit; Invitrogen) performed according to the manufacturer's instructions. Reverse transcription of all samples was carried out using the Superscript VILO kit (Invitrogen) according to the manufacturer's instructions, using 1 μg of total RNA as input for cDNA synthesis. Following this, cDNA was diluted 1:10 in ddH_2O for a total input of 5 ng into the quantitative PCR reaction, done using FastSybr (Applied Biosystems) and conducted on a Step-One ABI System (Applied Biosystems). Quantification of mRNA levels was accomplished using the standard curve method, with amplification of target mRNA and control genes in separate wells. Each sample was run in duplicate. The relative amount of mRNA in each well was calculated as the ratio between *Grn* mRNA (forward: 5′-CTGTAGTGCAGATGGGAAATCCTGCT-3′; reverse: 5′-GTGGCAGAGTCA GGACATTCAAACT-3′) and a normalization factor created using two control genes, *Usf1* (forward: 5′-CCTGTGGCGTGGCAGTCT-3′; reverse: 5′-TGCACGCCCACACTGTTT-3′) and *Paklip1* (forward: 5′-CCCCAAGTGGA GGGAAGTACA-3′; reverse: 5′-TGCCCAGCCGATAGACATC-3′) based on GeNorm (Vandesompele et al., 2002). Values are presented as % Floxed control.

2.4. Immunohistochemistry

Serial 25 μm floating sections were placed in net-well inserts and washed for 10 min in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched with 1% H_2O_2 for 45 min. After a 15 min wash in PBS with 0.1% Triton X (PBS-T), sections were blocked in 5% normal serum and 5% bovine serum albumin diluted in PBS-T, followed by overnight incubation shaking at room temperature in primary antibody diluted in 5% normal serum and PBS-T. After two 15 min washes in PBS-T, secondary antibody diluted in 1% normal serum and PBS-T was applied for 2 h shaking at room temperature. Sections were washed for 30 min in PBS before an amplification step was performed using an avidin–biotin–horseradish peroxidase complex kit (Vector Laboratories). Colorimetric detection was achieved with the peroxidase substrate kit Vector DAB (Vector Laboratories) according to the manufacturer's instructions. Sections were mounted by hand on onto glass slides (Fisherbrand Superfrost Plus) and dried overnight before being dehydrated through a series of alcohols and xylene, and cover-slipped with DEPEX (Electron Microscopy Sciences). Antibodies used were as follows: the neuronal marker NeuN (Chemicon, Millipore, 1:2000, mouse monoclonal), the microglia marker Iba1 (Wako; 1:2000, rabbit polyclonal), the astrocyte marker GFAP (Sigma; 1:2000, mouse monoclonal), and appropriate biotinylated secondary antibodies (Vector, 1:2000).

Immunofluorescent staining was performed by incubating mounted, 25 μm serial sections in PBS-T for 30 min, followed by 1 h in blocking solution (5% normal donkey serum in PBS-T) and subsequent overnight incubation at 4 °C with primary antibodies in 2% normal serum. Primary antibodies included NeuN (mouse monoclonal, Millipore, 1:1000), Iba1 (rabbit polyclonal, Wako, 1:1000), and Grn (sheep polyclonal, R&D

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