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Progranulin and Central Nervous System Disorders

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Preface

Progranulin (PGRN), which was identified as a sex steroid-inducible gene in the brain, is a protein also known as acrogranin, granulin/epithelin precursor, proepithelin, and PC cell-derived growth factor. It is an 88-kDa glycoprotein involved in masculinization of the brain during the perinatal period and facilitation of adult neurogenesis in the hippocampus. Recently, PGRN has been shown to promote neuronal survival and the regulation of inflammation in the brain, retina, and spinal cord. In other words, PGRN has variable effects on the central nervous system (CNS). There are various opinions about the neurotrophic and neuroprotective actions of PGRN depending on the circumstances. The aim of this book is to summarize the recent findings about the many actions of PGRN on the CNS in embryology, pathophysiology, and pharmacology.

PGRN is ubiquitously expressed in many cell types. In the CNS, it is produced by neurons, astrocytes, microglia, and endothelial cells. Furthermore, several stressors, such as hypoxia, glucose deprivation, acidosis, and oxidative stress, induce PGRN expression. These facts imply that PGRN plays important roles in homeostasis under various stresses. In fact, PGRN is a widely secreted growth factor regulating cell growth and survival, wound repair, and inflammation. Interestingly, PGRN may act as an autocrine neurotrophic factor because it is cotransported with brain-derived neurotrophic factor and secreted in an activity-dependent manner. There are also interesting research subjects in relation to intracellular trafficking of PGRN and its role in lysosome biogenesis and function. Once in the extracellular space, the potential fates of PGRN include cleavage into granulins by extracellular proteases or uptake into target cells via binding of its C-terminus to sortilin. Sortilin ultimately delivers PGRN via trafficking from endosomes to lysosomes, where it may be cleaved and/or degraded in a manner that regulates its intracellular levels. Lysosome and autophagy dysfunction are key players in neurodegenerative disease pathogenesis. PGRN may regulate the activities of lysosomal enzymes as a molecular chaperone and play a crucial role in maintaining cellular protein homeostasis. Therefore, it is natural that PGRN is heavily involved in neurodegenerative disorders.

A deeper understanding of the molecular and functional properties of PGRN would provide new insights for developing mechanism-based therapeutic approaches for multiple disorders, including neurodegenerative diseases in the CNS. Medications targeting the recovery of neuronal functions seem to be the most reasonable way for symptomatic improvement in patients with neurodegenerative diseases resulting from PGRN insufficiency. This book is dedicated to providing opportunities to increase the knowledge and understanding about how PGRN regulates lysosomal homeostasis and how PGRN has neuroprotective effects against inflammation and ischemia.

All authors are specialists of PGRN research, and most of the chapter authors have participated in the society for the study of PGRN in Japan. We especially thank all chapter authors for their valuable contributions. In this book, up-to-date information about the actions of PGRN has been explained in great detail. We believe that the contents of this book can be useful for a wide variety of readers with an interest in neurodegenerative research. At first, some readers may skip certain chapters, but that is no problem at all. We think that it is most important for researchers to make good use of the book to increase their knowledge. As long as the readers enjoy reading the parts that are of interest to them or are their favorite topics, that is all that is needed. We hope this publication will serve as a basis for future research about PGRN.

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Contents

Molecular and Functional Properties of Progranulin

Masugi Nishihara

Abstract We have identified progranulin (PGRN) gene as a sex steroid-inducible gene in the brain, which is involved in masculinization of the brain during the perinatal period and facilitation of adult neurogenesis in the hippocampus. PGRN was first reported in early 1990s by different groups as a protein containing seven and a half cysteine-rich granulin domains and having growth promoting properties involved in wound healing and tumorigenesis. Later it was found that mutations of the PGRN gene were associated with neurodegenerative and lysosomal storage diseases such as frontotemporal lobar degeneration and neuronal ceroid lipofuscinosis, respectively. We have also found that PGRN is located in lysosomes and deficiency of PGRN results in exacerbated neuroinflammatory responses and increased lysosomal biogenesis in microglia after traumatic brain injury. It is now recognized that PGRN may regulate activities of lysosomal enzymes as a molecular chaperone and play a crucial role in maintaining cellular protein homeostasis. Deeper understanding of molecular and functional properties of PGRN would provide new insights for developing mechanism-based therapeutic approaches for multiple disorders including cancers and neurodegenerative diseases.

Keywords Estrogen · Inflammation · Lysosome · Neurodegeneration · Neurogenesis · Progranulin · Tumorigenesis

Introduction

Our research groups have long been studying sex steroid actions in the brain, which are generally divided into three categories, i.e. organization, activation and protection. As shown in Fig. 1, organization takes place during perinatal period and involved in sexual differentiation of the brain, while activation and protection occur in the adulthood and involved in modulating sex behaviors/gonadotropin secretion

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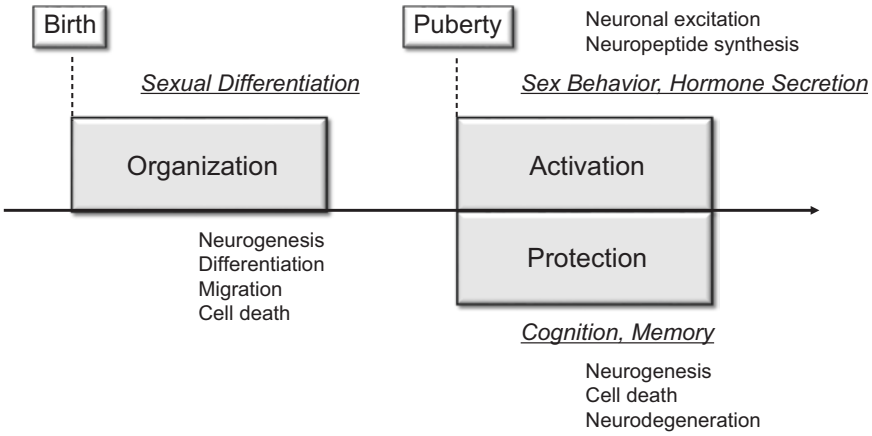


Fig. 1 Sex steroid actions in the brain, which are categorized as organization, activation and protection. During a perinatal period, sex steroids modulate neuronal proliferation, differentiation, migration and cell death, and thereby induce sexual differentiation of the brain (organization). In the adulthood, sex steroids elicit sexually dimorphic behaviors and hormone secretion pattern by modulating neuronal excitation and neuropeptide synthesis (activation). In addition, sex steroids facilitate adult neurogenesis and decrease neuronal cell death and neurodegeneration with a resultant maintenance of memories and cognitive function (protection)

and maintaining memories/cognitive function, respectively. Although sex steroid actions are exerted in general through new gene transcription and, hence, new protein translation, in the case of organization, sex steroids eventually affect neuronal proliferation, differentiation, migration and cell death in the developing brain. On the other hand, in the case of activation, sex steroids mainly induce neuronal excitation and neuropeptide synthesis. In addition, in the case of protection, sex steroids increase adult neurogenesis, while decrease neuronal cell death as well as neurodegeneration. We first tried to elucidate neuronal mechanisms involved in activational actions of sex steroids. To this end, we developed the recording system for the electrical activity of gonadotropin-releasing hormone (GnRH) pulse generator activity in the hypothalamus in unanesthetized rats and goats (Nishihara et al. 1991; Mori et al. 1991), which was an adaptation originally developed for rhesus monkeys by Knobil's group (Wilson et al. 1984). We found in both rats and goats that GnRH pulse generator activity declined during sex steroid-induced luteinizing hormone (LH) surge, which leads the concept that GnRH surge generator exists in the hypothalamus independent from the pulse generator (Nishihara et al. 1994, 1999).

Thus, it is now recognized that there are pulse and surge generators that mediate negative and positive feedback actions of sex steroids, respectively, on gonadotropin secretion in the female brain, while only a pulse generator exists in the male brain. Later, kisspeptin was identified as a potent stimulator of gonadotropin secretion, and there are two major populations of kisspeptin neurons in the hypothalamus, one in the arcuate nucleus and the other in the anteroventral periventricular nucleus, in the female brain, while only arcuate kisspeptin neurons are fundamentally functional

in the male brain. Kisspeptin neurons in the anteroventral periventricular nucleus are regarded to play a role as a surge generator and those in the arcuate nucleus as a pulse generator (Gottsch et al. 2004; Uenoyama et al. 2009). Thus, there are clear histological and functional differences between female and male brains in regulating gonadotropin secretion.

We then tried to elucidate molecular mechanisms involved in generating sex differences in the brain. Organizational actions are manifested by androgens derived from the testis after conversion to estrogens during fetal or perinatal periods (depending on species), which masculinize and defeminize the brain only in males, and the lack of these androgen actions develops default female type brain. To identify sex steroids-inducible genes involved in sexual differentiation of the brain, we used the cDNA subtraction method, which enables to isolate clones that are unequally expressed between two specimens. By means of this method, we have successfully isolated the progranulin (PGRN) gene, of which expression is enriched in the hypothalamus of male than female neonatal rats during the critical period of sexual differentiation of the brain (Suzuki et al. 1998). We confirmed that the expression of PGRN in the hypothalamus is indeed upregulated by sex steroids (Suzuki et al. 2001). To elucidate the role of PGRN in the developing brain, we tested the effect of the injection of the antisense oligodeoxynucleotide (ODN) complementary to PGRN mRNA in neonatal male rat brain, and found that subject animals that were treated with the antisense ODN had declined male sex behaviors after maturation, suggesting that PGRN is involved in mediating sex steroid actions organizing male type brain (Suzuki et al. 2000). Although PGRN had been already known molecule as described below, this is the first finding regarding the physiological role of PGRN in the central nervous system.

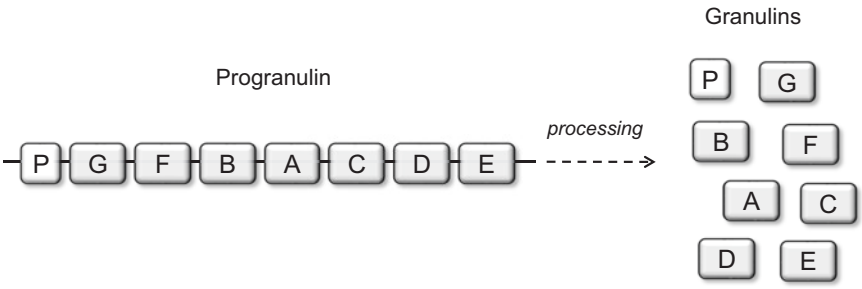
We then generated a line of mice with targeted disruption of the PGRN gene, and found that PGRN-deficient mice exhibited decreased ejaculation incidence and increased aggressiveness and anxiety as compared with wild-type mice (Kayasuga et al. 2007). These observations further support the notion that PGRN plays the organizational role in the neonatal brain by mediating sex steroid actions. This is also the first report regarding the production and phenotypic analyses of PGRN-deficient mice. Further, we found that enhancement of the adult neurogenesis in the dentate gyrus of the hippocampus by estrogen is at least partially mediated by PGRN (Chiba et al. 2007). Taken together, PGRN expression is enhanced by estrogen in both developing and adult brains, and it plays multifunctional roles, i.e. the masculinization in the developing brain (organizational action) and the maintenance of adult brain function (neuroprotective action) (Suzuki et al. 2009).

Structure of PGRN

In early 1990s, peptides derived from PGRN were identified by several groups from different tissues/cells and differently named. In 1990, Shoyab et al. purified two peptides from rat kidney having growth-modulating properties for human epidermal

carcinoma cells, and termed epithelin 1 and epithelin 2 (Shoyab et al. 1990). Bateman et al. also isolated in 1990 cysteine-rich peptides with molecular weights of approximately 6 kDa from human peripheral leukocytes and rat bone marrow, and called granulins (Bateman et al. 1990). Sequence analyses of amino acids and cDNA revealed that these peptides are derived from a single precursor protein, PGRN, which contains seven and a half tandem cysteine-rich granulin domains (granulins A to G and paragranulin) (Bhandari et al. 1992; Plowman et al. 1992). On the other hand, Gerton and colleagues identified in 1990 an acrosomal glycoprotein at around 67 kDa as a marker for acrosome development of the sperm from guinea pig testis, and named acrogranin (Anakwe and Gerton 1990), which was later found to be a guinea pig homologue of human and rat PGRN (Baba et al. 1993a). In addition, Serrero and colleagues reported in 1993 that a highly tumorigenic cell line, PC cell line, of mouse origin secrets an autocrine growth factor of 88 kDa glycosylated protein having growth promoting activity for 3T3 cells as well as PC cells, and termed PC cell-derived growth factor (or PCDGF), which also corresponds to a homologue of PGRN (Zhou et al. 1993). Collectively, these observations indicate that PGRN is ubiquitously expressed among tissues/cells of different species, and PGRN and its processed peptides, if not all, have growth regulatory properties.

Sequences of nucleotides of cDNA and deduced amino acids for human and rat granulin precursor (Bhandari et al. 1992, 1993), rat, mouse and human epithelin precursor (Plowman et al. 1992), mouse PCDGF precursor (Zhou et al. 1993), and guinea pig acrogranin (Baba et al. 1993a) have been independently reported. PGRN is encoded by *Grn* gene, and in mammalian genome, there is only one highly conserved *Grn* gene without other genes with high sequence homology (Palfree et al. 2015). In the mouse, *Grn* gene spans approximately 6.3 kbp containing 13 exons interrupted by 12 introns (Baba et al. 1993b). The cDNAs for human and mouse PGRN encode a polypeptide of 593 and 589 amino acids, respectively, including a 17 residue of putative signal sequence (Bhandari et al. 1992; Baba et al. 1993a). PGRN molecule is composed of seven and a half tandem repeats of the granulin module, which is characterized by a conserved pattern of 12 cysteine residues. Each granulin module is about 6 kDa and separated by short joining sequences, and could be released following proteolysis by, for example, elastase. The structure of human PGRN and amino acid sequences of each granulin peptide are shown in Fig. 2. The spatial conformation analysis revealed that the granulin modules adopt a compact fold of a parallel stack of β-hairpins stapled together by six disulfide bonds (Hrabal et al. 1996). According to Palfree et al. (2015), the granulin module arose in eukaryotic evolution, and PGRN is among the earliest extracellular regulatory proteins still employed by multicellular animals. Deeper understanding of molecular properties of PGRN would provide new insights for developing therapeutic means for multiple disorders, in which PGRN is involved.



Paragranulin: 18 TR**C**PDGQF**C**PVA**CC**LDPGGASYS**CC**RPLLD 47

Granulin A: 281 DVK**C**DMEVS**C**PDGYT**CC**RLQSGAWG**CC**PFTQAV**CC**EDHIH**CC**PAGFT**C**DTQKGT**C**E 336

Granulin B: 206 VM**C**PDARSR**C**PDGST**CC**ELPSGKYG**CC**PMPNAT**CC**SDHLH**CC**PQDTV**C**DLIQSK**C**L 261

Granulin C: 364 VP**C**DNVSS**C**PSSDT**CC**QLTSGEWG**CC**PIPEAV**CC**SDHQH**CC**PQGYT**C**VAEGQ**C**Q 417

Granulin D: 442 IG**C**DQHTS**C**PVGQT**CC**PSLGGSWA**CC**QLPHAV**CC**EDRQH**CC**PAGYT**C**NVKARS**C**E 496

Granulin E: 518 DVE**C**GEGHF**C**HDNQT**CC**RDNRQGWA**CC**PYRQGV**CC**ADRRH**CC**PAGFR**C**AARGTK**C**L 573

Granulin F: 123 AIQ**C**PDSQFE**C**PDFST**CC**VMVDGSWG**CC**PMPQAS**CC**EDRVH**CC**PHGAF**C**DLVHTR**C**I 179

Granulin G: 58 GGP**C**QVDAH**C**SAGHS**C**IFTVSGTSS**CC**PFPEAVA**C**GDGHH**CC**PRGFH**C**SADGRS**C**F 113

Fig. 2 Structure of human PGRN and amino acid sequence of each granulin peptide. PGRN is proteolytically cleaved to granulin peptides from granulin A to granulin G and paragranulin. Numbers are the position of amino acids. Cysteine residues are underlined

Receptors and Signaling Pathways

The mechanisms of action of PGRN have not yet been fully elucidated. As potential receptors for PGRN, several proteins including tumor necrosis factor receptors (TNFRs) (Tang et al. 2011), sortilin (Hu et al. 2010) and ephrin type-A receptor 2 (EPHA2) (Neill et al. 2016) have been proposed. PGRN exhibits comparable binding affinity for TNFR1 and TNFR2, and has higher affinity for them when compared to TNF-α (Tang et al. 2011). Since PGRN inhibits TNF-α binding to TNFR1 and TNFR2, PGRN may act as a physiological antagonist of TNF-α signaling, which may at least partially explain the anti-inflammatory actions of PGRN. Egashira et al. (2013) have also demonstrated that PGRN inhibits the binding of ^{125}I-labeled TNF-α to the surface of neutrophils isolated from rat peritoneal cavities and suppresses neutrophil chemotaxis induced by TNF-α. Interestingly, they have further shown that PGRN ameliorates neuronal injury induced by ischemia-reperfusion in mice, which may be due to the inhibition of neutrophil recruitment with a resultant reduction of nuclear factor-κB and matrix metalloproteinase-9 activation. Considering anti-inflammatory properties of PGRN, it is noteworthy to mention here that autoantibodies against PGRN have been detected in the serum of patients with different autoimmune diseases including psoriatic arthritis and inflammatory

bowel disease (Thurner et al. 2013, 2014). It was also shown that the protective effects of PGRN are inhibited by serum containing PGRN-autoantibodies by TNF-α-induced cytotoxicity assays. It is suggested from these observations that PGRN-autoantibodies in the serum might provide a proinflammatory environment in a subgroup of patients with autoimmune diseases, and could be used for a diagnostic and prognostic marker. Regarding TNFRs as potential receptors for PGRN, however, there is an opposing argument that PGRN does not bind TNFRs and is not a direct regulator of TNF-dependent signaling (Chen et al. 2013).

Sortilin is a trafficking protein that binds extracellular PGRN and conveys it to lysosomes rather than mediates signal transduction (Hu et al. 2010). It has been also reported that prosaposin (PSAP), the precursor of saposin peptides, interacts with PGRN and facilitates its lysosomal targeting in both biosynthetic and endocytic pathways via the cation-independent mannose 6-phosphate receptor (M6PR) and low density lipoprotein receptor-related protein 1 (LRP1) (Zhou et al. 2015). Sortilin and PSAP are thus regarded as two independent and complementary pathways for PGRN trafficking to lysosomes. Interestingly, it has been recently reported that retrograde signaling from PGRN released by Purkinje cells (PCs) to sortilin expressed on the membrane of climbing fibers (CFs) might strengthen and maintain the input from CFs to PCs in the developing cerebellum of infantile mice, thereby contributing to the selection of single CFs that survive synapse elimination (Uesaka et al. 2018). This finding may at least partially explain our previous observations that the density of dendrites of PCs in the molecular layer of the cerebellum was significantly higher in PGRN-deficient than in wild-type mice, though the number of the PCs was comparable between the genotypes (Matsuwaki et al. 2015). Since PGRN-deficient mice exhibited impairment of motor function as well, we suggested that PGRN is involved in the development of neuronal networks comprising PCs in the cerebellum, which may be prerequisite to exhibit normal motor function. On the other hand, Neill et al. (2016) reported that EPHA2, a member of the large family of ephrin receptor tyrosine kinases, is a functional signaling receptor for PGRN and that interaction of PGRN with EPHA2 causes intrinsic tyrosine kinase activation and downstream stimulation of mitogen-activated protein kinase (MAPK) and Akt, and in turn promotion of capillary morphogenesis. Although these studies provide new insights into the actions of PGRN, further studies are needed to elucidate the physiological receptors that transduce PGRN signaling.

Although functional receptors for PGRN are still elusive, it is known that several signal transduction pathways, especially those involved in cell proliferation and survival, are activated by PGRN. Granulin/epithelin precursor or PCDGF is shown to cause phosphorylation of shc and p44/42MAPK in the extracellular regulated kinase (ERK) pathway as well as phosphatidylinositol 3-kinase (PI3K) and Akt in the PI3K signaling pathway in 3T3 mouse embryo fibroblasts and human breast cancer MCF-7 cells (Zanocco-Marani et al. 1999; Lu and Serrero 2001; He et al. 2002). We have previously shown that PGRN enhances neural progenitor cell proliferation *in vitro* through the phosphorylation of glycogen synthase kinase 3β (GSK3β), which is dependent on PI3K activity (Nedachi et al. 2011). PGRN also causes phosphorylation of the receptor tyrosine kinase and activation of downstream MAPK and

PI3K/Akt pathways in human umbilical vein endothelial cell as mentioned above (Neill et al. 2016).

Regulation of PGRN Expression

PGRN is expressed in a wide variety of tissues/cells and cancers. The high expression levels are observed in the spleen, adrenal gland and several reproductive organs including the placenta, ovary and epididymis (Bhandari et al. 1993). Several cancer cells express PGRN, and PGRN contributes tumorigenesis of breast cancer (Lu and Serrero 2000), ovarian carcinoma (Jones et al. 2003), glioblastoma (Liau et al. 2000) and multiple myeloma (Wang et al. 2003). In the brain, PGRN is mainly expressed in the cingulate and piriform cortices, the pyramidal cell layer and dentate gyrus of the hippocampus, the amygdala, the ventromedial and arcuate nuclei of the hypothalamus and the Purkinje cell layer in the cerebellum (Matsuwaki et al. 2011). As mentioned above, PGRN expression in the brain is enhanced by estrogen in both the perinatal period and adulthood. In addition, it has been also shown that the expression of PCDGF mRNA and protein in MCF-7 cells (human breast cancer cells) is stimulated by estrogen (Lu and Serrero 1999). Although there are no complete palindromic estrogen responsive elements (EREs), many ERE half-palindromic motifs are located in the 5′-upstream region of the mouse *Grn* gene (our unpublished observation). Since ERE half sites have been shown to be able to synergistically mediate estrogen-induced transcriptional activation (Kato et al. 1992), estrogen may stimulate *Grn* gene expression through these ERE half sites.

The expression of PGRN is upregulated in the inflammatory cells during wound healing (He et al. 2003). In the brain, traumatic brain injury enhanced PGRN expression in CD68-positive microglia (Tanaka et al. 2013a). We have previously reported that the mouse *Grn* gene has two possible coordinated lysosomal expression and regulation (CLEAR) sequences that bind to transcription factor EB (TFEB), a master regulator of lysosomal genes (Sardiello et al. 2009), in the promoter region and that PGRN is colocalized with Lamp1, a lysosomal marker. These observations suggest that PGRN is a member of lysosomal proteins and plays a role in regulating cellular protein homeostasis in lysosomes as well as extracellular regulatory proteins (Tanaka et al. 2013b). The expression of PGRN is also increased in activated microglia by lipopolysaccharide (LPS)-induced acute immune stress, which in turn attenuates neuroinflammation (Ma et al. 2017b). Kanazawa et al. (2015) showed that, following experimental acute focal cerebral ischemia in rats, increased levels of PGRN expression were observed in microglia within the ischemic core, and in viable neurons as well as endothelial cells within the ischemic penumbra. They further demonstrated that PGRN could protect against acute focal cerebral ischemia by a variety of mechanisms including attenuation of blood-brain barrier disruption, neuroinflammation suppression, and neuroprotection. Taken together, PGRN expression is upregulated in the brain and plays a neuroprotective role following brain damages. In addition, we have recently found that PGRN

expression is also increased in the skeletal muscle following muscle injury induced by the injection of cardiotoxin and modulates the recovery process as described below (Sugihara et al. 2018).

The PGRN expression during myoblast fusion is a consequence of the binding of MyoD, a transcription factor for myogenic differentiation, to several E-box (CANNTG) sequences in the 5′-flanking regulatory region of *Grn* gene, followed by transcription (Wang et al. 2012). Since the regulation of myotube formation by PGRN is mediated by the anti-myogenic factor JunB, which is upregulated following PGRN stimulation, they suggest that PGRN, JunB and MyoD form a regulatory loop and act in concert in the course of myogenesis. Matsubara et al. (2012) reported that PGRN is secreted from the adipose tissue as an adipokine and the expression in the adipocytes is increased by TNF-α and glucocorticoid. They also showed that PGRN mediates high fat diet-induced insulin resistance and obesity through production of IL-6 in adipose tissue, and proposed that PGRN may be a promising therapeutic target for obesity.

PGRN as a Growth Factor

PGRN molecule is preceded by a signal sequence and, hence, supposed to be a secretory peptide. As mentioned above, PGRN and/or granulins have long been regarded as an extracellular growth-modulating peptide(s). Both epithelin 1 and epithelin 2 inhibit the growth of A431 cells, which are derived from a human epidermal carcinoma (Shoyab et al. 1990). In addition, epithelin 1 stimulates the proliferation of murine keratinocytes, while epithelin 2 inhibits the epithelin 1-elicited growth of these cells. PGRN is suggested to be involved in early embryogenesis as well. Gerton and colleagues found that acrogranin (epithelin/granulin precursor) is expressed in preimplantation mouse embryos and secreted into the surrounding medium (Díaz-Cueto et al. 2000). Blocking the function of acrogranin by anti-acrogranin antibody significantly inhibited the development of eight-cell embryos to the blastocyst and decreased embryo cell numbers, while exogenous acrogranin added to the culture accelerated the time for the onset of cavitation and increased the number of trophectoderm cells.

The role of PGRN in wound healing was described by Bateman's group (He et al. 2003). In murine transcutaneous puncture wounds, PGRN is expressed in the inflammatory cells, dermal fibroblasts and endothelia, and addition of PGRN increases the accumulation of neutrophils, macrophages, blood vessels and fibroblasts in the wound. PGRN acts directly on isolated dermal fibroblasts and endothelial cells to promote division, migration and the formation of capillary-like tubule structures. Zhu et al. (2002) reported that epithelins inhibit the growth of epithelial cells but induce them to secrete the neutrophil attractant IL-8, while proepithelin blocks neutrophil activation by TNF, preventing the release of oxidants and proteases. Secretory leukocyte protease inhibitor (SLPI) and proepithelin form complexes, preventing elastase from converting proepithelin to epithelins. In addi-

tion, proepithelin restores the wound-healing defect in SLPI null mice. From these observations, they propose that SLPI/elastase act via proepithelin/epithelins to operate a switch at the interface between innate immunity and wound healing. Following retinal damage using *N*-methyl-*N*-nitrosourea in mice, PGRN promotes retinal precursor cell proliferation and the photoreceptor differentiation through the hepatocyte growth factor receptor signaling pathway (Kuse et al. 2016), while PGRN deficiency causes the retinal ganglion cell loss subsequent to the activation of astrocytes during retinal development (Kuse et al. 2017).

Interestingly, PCDGF is expressed in MCF-7 human breast cancer cells, of which expression is upregulated by estrogen and mediates a mitogenic effect of it on MCF-7 cells (Lu and Serrero 1999, 2001). We have shown that estrogen stimulated adult neurogenesis in the dentate gyrus of the hippocampus in rats, which was associated with an increase in PGRN gene expression (Chiba et al. 2007). In addition, an increase in proliferation of neural progenitor cells *in vitro* by estrogen was blocked by the addition of anti-PGRN antibody into the medium. These observations suggest that a mitogenic effect of estrogen on adult neurogenesis in rat hippocampus is also mediated by PGRN. Thus, PGRN seems to mediate sexual differentiation of the brain during neonatal period and neurogenesis in the hippocampus during adulthood, both of which are the consequence of estrogen actions in the brain. We have also shown that PGRN enhances proliferation of neural progenitor cells derived from PGRN-deficient mice, but not from wild-type mice, probably due to high expression levels of endogenous PGRN (Nedachi et al. 2011). Regarding adult neurogenesis, we have shown that the expression of PGRN in the hippocampus is upregulated by voluntary exercise, and mediates exercise-induced increases in the number of proliferating cells in the dentate gyrus of the hippocampus (Asakura et al. 2011). In addition, PGRN attenuates a decline of hippocampal neurogenesis by LPS-induced acute immune stress (Ma et al. 2017b), but not by aging (Ma et al. 2017a), though PGRN suppresses neuroinflammation in both cases.

PGRN is expressed in the skeletal muscle as well, and a previous study showed that PGRN promoted myotube hypertrophy through PI3K/Akt/mTOR pathway, using immortalized mouse myoblast cell line C2C12 cells (Hu et al. 2012). On the other hand, there is a report showing that PGRN inhibits myotube formation *in vitro* and that knockdown of PGRN enhances myogenesis in neonatal mice (Wang et al. 2012). These findings suggest that PGRN may play a role in the skeletal muscle, but its action in myogenesis *in vivo* is still controversial. We have recently found the prolonged persistence of macrophages at the late phase of regeneration in PGRN-deficient mice following muscle injury induced by cardiotoxin (Sugihara et al. 2018). These macrophages were suggested to be M2 macrophages since this was accompanied with an increased CD206 expression. We also observed muscle hypertrophy in PGRN-deficient mice at the late stage of muscle regeneration. Since M2 macrophages are known to have a role in maturation of myofibers, the muscle hypertrophy observed in PGRN-deficient mice may be due to the presence of increased number of M2 macrophages following muscle injury. Our results suggest that PGRN plays a role in the regulation of kinetics of macrophages for the systematic progress of muscle regeneration.

PGRN as a Lysosomal Protein

In the human brain, haploinsufficiency of PGRN is one of the major factors causing frontotemporal lobar degeneration (FTLD) (Baker et al. 2006; Cruts et al. 2006), which is characterized by ubiquitinated cytoplasmic inclusions containing TAR DNA binding protein 43 (TDP-43) (Arai et al. 2006; Neumann et al. 2006). Patients with a homozygous mutation in the *GRN* gene present with neuronal ceroid lipofuscinosis (NCL), a group of neurodegenerative lysosomal storage disorders (Smith et al. 2012). Götzl et al. (2014) also revealed that FTLD patients due to PGRN deficiency have NCL-like pathology. We have already shown that PGRN-deficient mice developed age-associated abnormal intraneuronal ubiquitin-positive autofluorescent lipofuscin with focal neuronal loss and gliosis, suggesting that PGRN plays a key role in maintaining neuronal function and survival during aging (Ahmed et al. 2010). Additionally, an association between PGRN insufficiency and Gaucher disease (GD), the most common lysosomal storage disease, has recently proposed (Jian et al. 2016b). These reports suggest that disorders derived from PGRN deficiency occur associated with lysosomal dysfunction.

We have previously shown that PGRN is produced in microglia positive for CD68, a member of the lysosome-associated membrane protein (Lamp) family, and suppresses excessive inflammatory responses after traumatic brain injury (TBI) in mice (Tanaka et al. 2013a). As schematically illustrated in Fig. 3, PGRN is localized in lysosomes of activated microglia and involved in the activation of mammalian

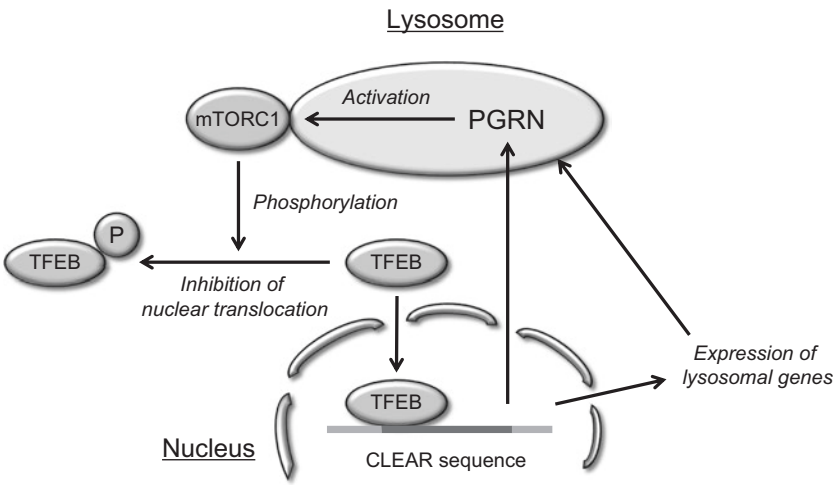


Fig. 3 Schematic illustration of the effect of PGRN on lysosomal biogenesis. TFEB, a master regulator of lysosomal genes, binds to the CLEAR sequence of DNA and activates transcription of lysosomal genes. PGRN is localized in lysosomes and involved in the activation of mTORC1, which inhibits translocation of TFEB to the nucleus by phosphorylation, thereby decreasing lysosomal biogenesis. PGRN has CLEAR sequences in its promoter region, and hence is a member of lysosomal proteins

target of rapamycin complex 1 (mTORC1). Activated mTORC1 then inhibits nuclear translocation of TFEB, which activates transcription of lysosomal genes by binding to the CLEAR sequence of DNA, by phosphorylation. Since PGRN gene has also CLEAR sequences in its promoter region as mentioned above, there seems to be a negative feedback loop in the biogenesis of lysosomes through PGRN. Thus, the deficiency of PGRN leads to increased nuclear translocation of TFEB with a resultant increase in lysosomal biogenesis in activated microglia and exacerbated neuronal damage after TBI (Tanaka et al. 2013b). Aged PGRN-deficient mice present with NCL-like pathology as well as TDP-43 aggregates in the thalamus, where a particular vulnerability has been reported in NCL model mice (Cooper 2010). Since aggregates of p62, which is selectively degraded by the autophagy-lysosomal system, were observed in neuronal and glial cells in the thalamus, it is also suggested that these pathological changes in the thalamus are likely a result of lysosomal dysfunction (Tanaka et al. 2014).

We have further shown that secreted PGRN was incorporated into cells via sortilin or M6PR, and facilitated the acidification of lysosomes (Tanaka et al. 2017). Lysosomal acidification is essential for digestive function and efflux of digested materials (Mindell 2012). The change of PGRN levels led to a cell-type-specific increase of insoluble TDP-43, and in the brain tissue of FTLD-TDP patients with PGRN deficiency, cathepsin D (CTSD) and phosphorylated TDP-43 accumulated in neurons. These studies provide new insights into the physiological function of PGRN as a lysosomal protein and the role of PGRN insufficiency in the pathogenesis of neurodegenerative and lysosomal storage diseases. Besides the brain, we have also shown that PGRN and/or granulins bind to CpG-ODNs and interact with Toll-like receptor 9 as a cofactor in endolysosomal compartments of peripheral macrophages, thereby accelerating the production of proinflammatory cytokines and eventually contributing innate immunity (Park et al. 2011). It may be worth mentioning here that PGRN has been identified as acrogranin in the acrosome of sperm as mentioned above, which contains proteases involved in acrosomal reaction during fertilization and is regarded as a kind of lysosome in sperm (Baba et al. 1993a).

Regarding GD, which is caused by mutations in *GBA1* encoding of β-glucocerebrosidase (GCase), Jian et al. (2016b) showed that the four single nucleotide polymorphism (SNP) sites in *GRN* gene are associated with a decrease in serum PGRN levels and exhibit significantly higher frequency in GD patients. In addition, both ovalbumin-challenged and aged PGRN-deficient mice develop GD-like phenotypes. They further revealed that PGRN plays a role as a co-chaperone for the lysosomal localization of GCase through linking GCase to heat shock protein 70 (HSP70), which is regarded as a highly conserved molecular chaperone (Jian et al. 2017). The association between PGRN and GCase requires C-terminus of PGRN containing granulin E domain (GrnE). A PGRN-derived protein consisting of 98 amino acids of C-terminal PGRN, referred to as Pcgin, effectively ameliorate the disease phenotype in GD patient fibroblasts and PGRN-deficient mice (Jian et al. 2016a). Interestingly, Beel et al. (2017) also showed recently that PGRN functions as a chaperone molecule of lysosomal enzyme CTSD to stimulate axonal

outgrowth, and that the interaction between PGRN and CTSD is mediated by GrnE, which maintains proper proteolytic capacity of CTSD. PGRN or granulin(s) therefore may regulate activities of multiple lysosomal enzymes as a molecular chaperone, and play a crucial role in modulating degradation of aggregated proteins, thereby maintaining proper protein homeostasis in the cell. This notion would extend potential therapeutic targets of PGRN and related granulin peptides.

Conclusion

PGRN is a highly conserved molecule among mammalian species without other molecules with high sequence homology, and consists of unique cysteine rich granulin domains. PGRN/granulins have been identified from different cells/tissues and differently termed, which indicates that they are ubiquitously expressed and play multifunctional roles. PGRN is first regarded as a secretory protein having growth modulatory properties, but is now also regarded as a lysosomal protein that maintains proper cellular protein homeostasis and has neuroprotective properties. In the brain, as summarized in Fig. 4, during a perinatal period, PGRN expression is

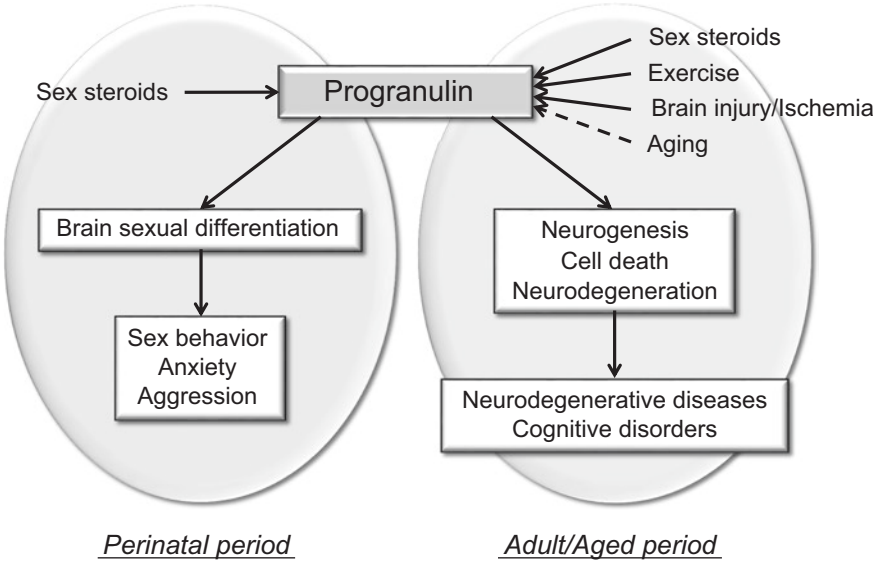


Fig. 4 Summary of the PGRN actions in the brain. During a perinatal period, PGRN expression is upregulated in the hypothalamus by sex steroids and involved in sexual differentiation of the brain, which affects sexually dimorphic behaviors including sex behaviors, anxiety and aggression afterwards. In the adulthood, PGRN expression is upregulated by exercise and brain injury/ischemia as well as sex steroids, but declines with age. PGRN facilitates adult neurogenesis while inhibits neuronal cell death and neurodegeneration, thereby preventing neurodegenerative diseases and cognitive disorders

upregulated in the hypothalamus by sex steroids and involved in sexual differentiation (masculinization/defeminization) of the brain, which affects sexually dimorphic behaviors including sex behaviors, anxiety and aggression after maturation. On the other hand, in the adulthood, PGRN expression is increased by exercise and brain injury/ischemia as well as sex steroids, but declines with age. PGRN facilitates adult neurogenesis, while attenuates neuronal cell death and neurodegeneration, thereby preventing neurodegenerative diseases and cognitive disorders with a resultant maintenance of higher brain functions. PGRN has been implicated in many kinds of disorders and thus may be a promising therapeutic target for multiple diseases such as cancers, neurodegenerative and lysosomal diseases, ischemic stroke, insulin resistance and retinal degenerating diseases.

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Progranulin as a Potential Biomarker of Central Nervous System Disease

Akio Kimura, Masao Takemura, and Takayoshi Shimohata

Abstract Progranulin is a cysteine-rich secreted protein initially identified as a growth factor. Progranulin has been implicated in multiple biological and pathological processes, including tumorigenesis, inflammation, neurodegeneration and lysosomal function. Loss of one allele of the progranulin gene (*GRN*) leads to frontotemporal lobar degeneration (FTLD). *GRN* null mutations cause haploinsufficiency, leading to a significant decrease in progranulin protein levels in the plasma, serum and cerebrospinal fluid (CSF) of carriers. Recently, several reports have shown that plasma progranulin levels predict *GRN* mutation status in patients with FTLD and asymptomatic family members. Thus, the concentration of circulating progranulin is a useful biomarker for screening *GRN* mutation carriers. Interestingly, there are also conditions in which expression of progranulin is increased. For example, progranulin is highly overexpressed in aggressive cancer cell lines and tissue specimens from various malignancies. Furthermore, progranulin expression in tumor and serum samples correlates with pathological grading and prognosis in several types of cancer. In the central nervous system (CNS), progranulin is often highly expressed in gliomas. Recently, we reported increased progranulin levels in the CSF of patients with CNS lymphomas and carcinomas with CNS metastasis. Accordingly, CSF progranulin levels may be useful as a diagnostic and monitoring marker for CNS metastases of lymphomas and carcinomas. Progranulin is also associated with various autoimmune diseases. For example, in rheumatoid arthritis and systemic lupus erythematosus, progranulin serum levels positively correlate with disease activity. Several reports also suggest an association with autoimmune CNS diseases, including multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD). Increased CSF progranulin levels are observed in the acute

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phase of these diseases. Additionally, although still controversial, increased progranulin levels appear to be associated with remission of symptoms in MS and NMOSD. Therefore, progranulin may be a promising therapeutic agent and useful biomarker of CNS diseases, including *GRN*-related neurodegenerative diseases, malignancies, and autoimmune neurological disorders.

Keywords Biomarker · Carcinoma · Central nervous system · Frontotemporal lobar degeneration · Malignancy · Malignant lymphoma · Metastasis · Multiple sclerosis · Neuromyelitis optica spectrum disorder

Introduction

Progranulin is a secreted glycosylated protein with critical functions in numerous biological and pathological processes, including cell growth, tumorigenesis, wound healing, inflammation, immunity, infection, and diabetes (Cenik et al. 2012; Eriksen and Mackenzie 2008; Jian et al. 2013a; Toh et al. 2011; Abella et al. 2017). In the central nervous system (CNS), progranulin acts as a neurotrophic and neuroprotective factor. Recently, changes in progranulin expression that are related to pathological conditions have been reported in various neurological diseases (Fig. 1). Mutations in the progranulin gene (*GRN*) were recently linked to certain forms of frontotemporal lobar degeneration (FTLD) (Baker et al. 2006; Cruts et al. 2006; Gass et al. 2006). The *GRN*-related form of FTLD is neuropathologically characterized by the appearance of neuronal inclusions containing ubiquitinated and fragmented TAR DNA binding protein-43 (TDP-43). Measurement of progranulin in blood and cerebrospinal fluid (CSF) can identify FTLD patients and asymptomatic carriers of *GRN* mutations, with progranulin haploinsufficiency leading to decreased

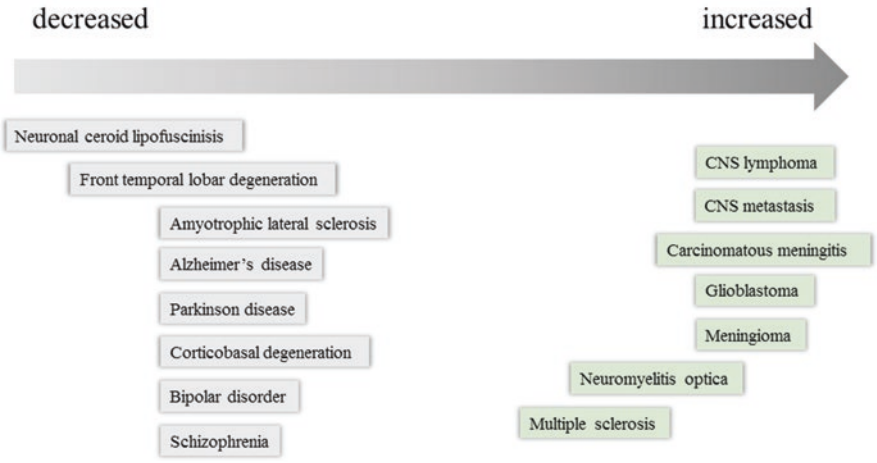

Fig. 1 Progranulin expression levels in central nervous system diseases

progranulin levels (Ghidoni et al. 2008, 2012a, b; Finch et al. 2009; Sleegers et al. 2009; Carecchio et al. 2011; Galimberti et al. 2018).

In addition to progranulin deficiency, there are conditions in which protein expression is increased. Progranulin levels in biological fluids are generally low, but are upregulated in the inflammatory state, strongly supporting its use as a biomarker of disease onset and progression in several pathologies (Abella et al. 2017). Progranulin stimulates cell division and promotes tumor formation (Serrero 2003; Ong and Bateman 2003; Serrero and Ioffe 2003), and it is highly expressed in aggressive cancer cell lines and many malignancies. Changes in circulating progranulin levels have been observed in breast cancer (Koo et al. 2012), ovarian cancer (Han et al. 2011), hematological malignancies (Göbel et al. 2013; Yamamoto et al. 2017), and non-small-cell lung cancer (Edelman et al. 2014), as assessed by enzyme immunoassay (EIA). Therefore, progranulin may have potential as a prognostic biomarker of malignancy recurrence.

Progranulin is also associated with the pathophysiology of several autoimmune diseases. Progranulin binds to tumor necrosis factor (TNF) receptors (TNFRs), and disrupts TNFα–TNFR interactions (Liu and Bosch 2012; Jian et al. 2013b; Tang et al. 2011). Progranulin-deficient mice are susceptible to collagen-induced arthritis, while administration of progranulin alleviates inflammatory arthritis (Tang et al. 2011). Moreover, there are several reports demonstrating significantly higher concentrations of serum progranulin in autoimmune diseases (including rheumatoid arthritis [RA] and systemic lupus erythematous [SLE]), compared with healthy controls (Tanaka et al. 2012; Yamamoto et al. 2014).

In this chapter, we summarize recent advances on the use of progranulin as a potential biomarker of CNS diseases, including malignancies, and neurodegenerative and autoimmune neurological disorders.

Progranulin as a Biomarker of Neurodegenerative Diseases

Progranulin was first reported as a growth factor associated with tumor growth (He and Bateman 1999). In the CNS, progranulin functions as a neurotrophic and neuroprotective factor (Chitramuthu et al. 2017), and recent studies show that *GRN* mutations cause several neurodegenerative diseases. The first *GRN* mutations were discovered in FTLD families with ubiquitin- and TDP43-positive pathologies (Baker et al. 2006; Cruts et al. 2006; Gass et al. 2006). While heterozygosity for the mutations results in FTLD, homozygosity leads to neuronal ceroid lipofuscinosis, a lysosomal storage disease (Smith et al. 2012; Almeida et al. 2016). The clinical symptoms associated with FTLD are diverse, including behavioral and personality changes, language disorders of expression and comprehension, cognitive impairment, and occasionally, motor neuron disease (McKhann et al. 2001). Intriguingly, missense *GRN* mutations are also observed in patients with clinically diagnosed Alzheimer's disease (Perry et al. 2013) and amyotrophic lateral sclerosis (ALS)

(Schymick et al. 2007). Thus, patients with *GRN* mutations can present with a variety of neurodegenerative diseases and a broad spectrum of clinical phenotypes.

GRN null mutations cause protein haploinsufficiency, leading to a significant reduction in progranulin levels in the plasma, serum and CSF of mutation carriers (Ghidoni et al. 2008, 2012a, b; Finch et al. 2009; Sleegers et al. 2009; Carecchio et al. 2011; Galimberti et al. 2018). The measurement of circulating progranulin levels enables screening of *GRN* mutation carriers quickly and inexpensively. Several reports show that plasma progranulin levels predict *GRN* mutation status in FTLD patients and asymptomatic family members (Ghidoni et al. 2008, 2012a; Finch et al. 2009; Galimberti et al. 2018). Finch et al. investigated progranulin levels in plasma samples from FTLD patients, including symptomatic and asymptomatic relatives of patients with *GRN* mutations (Finch et al. 2009). All FTLD patients with *GRN* mutations showed significantly reduced levels of progranulin in plasma, to approximately one-third of the levels observed in non-*GRN* carriers and control individuals. These researchers also found low progranulin levels in asymptomatic *GRN* mutation carriers. Galimberti et al. investigated whether plasma progranulin levels are predictors of *GRN* null mutations in FTLD family members in a cohort including FTLD patients, asymptomatic carriers, and non-carriers (Galimberti et al. 2018). They found that plasma progranulin levels in FTLD patients and asymptomatic carriers were significantly decreased compared with non-carriers. At a threshold of 61.55 ng/mL, the test showed a sensitivity of 98.8% and a specificity of 97.5% for predicting the presence of *GRN* null mutations, independent of symptoms. Thus, circulating progranulin levels may be a reliable biomarker, with high sensitivity and specificity, for the diagnosis and early detection of *GRN*-related neurodegenerative diseases. Measuring circulating progranulin levels may become an indispensable tool for preventing or delaying the onset of *GRN*-related neurodegenerative diseases in the near future.

Progranulin as a Biomarker of CNS Malignancies

Recent studies suggest that progranulin may be a potential clinical biomarker of various malignancies. Progranulin is associated with cell proliferation, migration, invasion, malignant transformation, angiogenesis, resistance to anticancer drugs, and immune evasion (Arechavaleta-Velasco et al. 2017). Progranulin is highly expressed in aggressive cancer cell lines and specimens from many malignancies (Table 1) (Serrero 2003; Serrero and Ioffe 2003; Han et al. 2011; Göbel et al. 2013; Yamamoto et al. 2017; Edelman et al. 2014; Frampton et al. 2012; Kim et al. 2010, 2012; Lovat et al. 2009; Selmy et al. 2010; Li et al. 2012; Tkaczuk et al. 2011; Lu et al. 2014; Wei et al. 2015a; Yang et al. 2015; Chen et al. 2008; Wang et al. 2012; Bandey et al. 2015; Ho et al. 2008; Cuevas-Antonio et al. 2010; Matsumura et al. 2006; Pan et al. 2004; Donald et al. 2001). Regardless of tumor type, progranulin is overexpressed in cancer cells and has growth-promoting and chemoresistant actions. In patients with malignancies, increased circulating progranulin levels have been

Table 1 Malignancies showing enhanced progranulin expression

	Malignancy	Specimen	References
1	Biliary tract carcinoma	Cell line, serum, tumor tissue	Frampton et al. (2012) and Kim et al. (2012)
2	Bladder cancer	Tumor tissue, urine	Lovat et al. (2009) and Selmy et al. (2010)
3	Breast cancer	Tumor tissue, serum	Serrero (2003), Serrero and Ioffe (2003), Li et al. (2012), and Tkaczuk et al. (2011)
4	Cervical cancer	Cell line, tumor tissue	Lu et al. (2014) and Wei et al. (2015a)
5	Chronic lymphocytic leukemia	Plasma	Göbel et al. (2013)
6	Colorectal cancer	Tumor tissue	Yang et al. (2015)
7	Esophageal squamous cell carcinoma	Tumor tissue	Chen et al. (2008)
8	Glioblastoma	Cell line, serum, tumor tissue	Wang et al. (2012) and Bandey et al. (2015)
9	Hepatocellular carcinoma	Tumor tissue	Ho et al. (2008)
10	Malignant lymphoma	Serum, tumor tissue	Yamamoto et al. (2017)
11	Meningioma	Tumor tissue	Kim et al. (2010)
12	Non-small-cell lung cancer	Tumor tissue, serum	Edelman et al. (2014)
13	Ovarian cancer	Tumor tissue, plasma	Han et al. (2011) and Cuevas-Antonio et al. (2010)
14	Uterine leiomyosarcoma	Tumor tissue	Matsumura et al. (2006)
15	Prostate cancer	Tumor tissue	Pan et al. (2004)
16	Renal cell carcinoma	Tumor tissue	Donald et al. (2001)

observed by EIA. Moreover, increased circulating progranulin levels correlate with pathological grading and prognosis in several types of cancer (Table 2) (Koo et al. 2012; Han et al. 2011; Göbel et al. 2013; Yamamoto et al. 2017; Edelman et al. 2014; Kim et al. 2010, 2012; Selmy et al. 2010; Wang et al. 2012; Bandey et al. 2015; Ho et al. 2008; Cuevas-Antonio et al. 2010; Donald et al. 2001; Serrero et al. 2012; Li et al. 2011; Carlson et al. 2013).

In the CNS, progranulin is often highly expressed in gliomas (Wang et al. 2012; Bandey et al. 2015). Progranulin plays a role in astrocytoma progression and is a prognostic biomarker for glioblastoma, with overexpression predicting decreased survival (Wang et al. 2012). Progranulin is overexpressed in tumors from patients with glioblastoma multiforme, and is associated with tumorigenicity and temozolomide resistance (Bandey et al. 2015). It is also implicated in the growth of intracranial meningioma (Kim et al. 2010). Recently, we reported that increased CSF progranulin levels are found in patients with CNS lymphomas (primary and secondary CNS lymphoma) and carcinomas with CNS metastasis (carcinomatous meningitis and brain metastasis) (Kimura et al. 2018). We examined CSF progranulin levels in various CNS diseases by EIA. Specifically, we compared progranulin

Table 2 Malignancies with an association between progranulin level and pathological grading or prognosis

	Malignancy	Specimen	References
1	Biliary tract carcinoma	Tumor tissue	Kim et al. (2012)
2	Bladder cancer	Urine	Selmy et al. (2010)
3	Breast cancer	Tumor tissue, serum	Koo et al. (2012), Serrero et al. (2012), and Li et al. (2011)
4	Chronic lymphocytic leukemia	Plasma	Göbel et al. (2013)
5	Glioblastoma	Tumor tissue	Wang et al. (2012) and Bandey et al. (2015)
6	Hepatocellular carcinoma	Tumor tissue	Ho et al. (2008)
7	Malignant lymphoma	Serum	Yamamoto et al. (2017)
8	Meningioma	Tumor tissue	Kim et al. (2010)
9	Non-small-cell lung cancer	Tumor tissue	Edelman et al. (2014)
10	Ovarian cancer	Tumor tissue, plasma	Han et al. (2011), Cuevas-Antonio et al. (2010), and Carlson et al. (2013)
11	Renal cell carcinoma	Tumor tissue	Donald et al. (2001)

levels among disease groups in CSF samples from 230 patients, including 18 with lymphomas (12 with CNS metastasis and 6 without CNS metastasis), 21 with carcinomas (10 with CNS metastasis and 11 without CNS metastasis), and 191 control patients with non-cancer neurological diseases. Median CSF progranulin levels were significantly higher in the lymphoma with CNS metastasis group compared with the lymphoma without CNS metastasis and control non-cancer groups. Additionally, levels were also significantly higher in the carcinoma with CNS metastasis group compared with the carcinoma without CNS metastasis and control non-cancer groups, except for patients with infectious neurological disorders (Fig. 2). Importantly, increased CSF progranulin levels were observed in lymphomas and carcinomas with metastasis regardless of tumor type. Using receiver operator characteristic (ROC) curves, we determined the suitability of CSF progranulin as a biomarker for lymphomas and carcinomas with CNS metastasis. The area under the ROC curve (AUC) was 0.969 for differentiating lymphoma with CNS metastasis (compared with lymphoma without CNS metastasis and non-cancer neurological diseases), and 0.918 for differentiating carcinoma with CNS metastasis (compared with carcinoma without CNS metastasis and non-cancer neurological diseases) (Fig. 3). These findings are clinically important because diagnosing CNS metastases can be difficult in patients with lymphomas and carcinomas as well as in those with histories of these diseases and whose neurological symptoms (such as headache, gait disturbance, sensory disturbance, and cognitive impairment) are also observed in other inflammatory and non-inflammatory neurological diseases. Diagnosis is also difficult in patients with lymphomas and carcinomas without CNS metastasis with paraneoplastic neurological syndromes or side effects of chemotherapy.

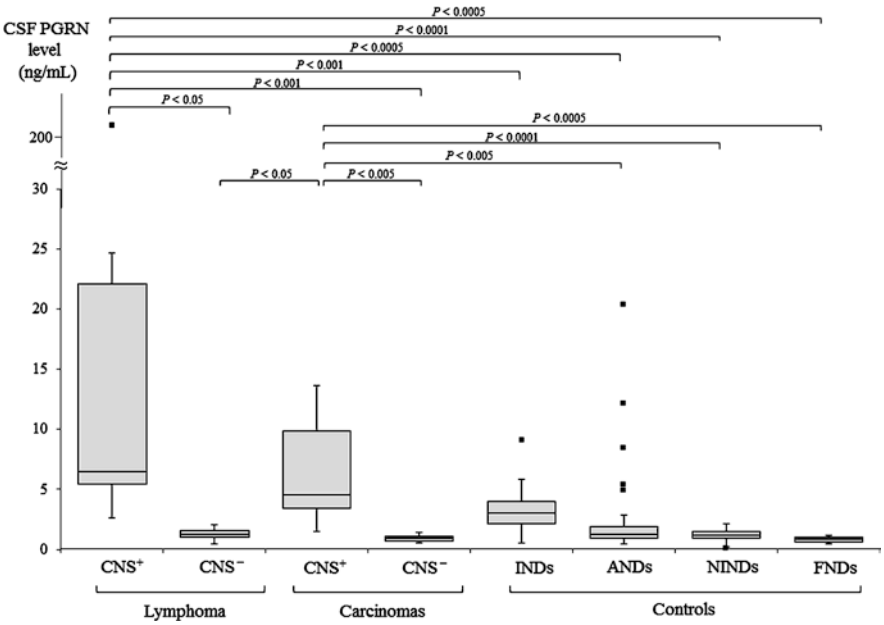

Fig. 2 Progranulin levels in cerebrospinal fluid (CSF PGRN) of patients with lymphoma and carcinoma with and without central nervous system (CNS) metastasis. CSF PGRN levels were significantly higher in patients with lymphoma and carcinoma with CNS involvement (CNS+) compared with those without CNS involvement (CNS−), as well as controls consisting of patients with non-cancer neurological diseases (such as autoimmune neurological disorders [ANDs], functional neurological disorders [FNDs], infectious neurological disorders [INDs], and non-inflammatory neurological disorders [NINDs]). Black dots: outliers

Numerous potential biomarkers for CNS malignancies have been reported. However, none are currently in clinical use for monitoring CNS metastasis (Berghoff et al. 2013). Diagnosis of CNS metastasis is usually based on brain magnetic resonance imaging studies and cytological examinations of CSF, but these methods have limited sensitivity and specificity. We therefore proposed that measuring CSF progranulin levels may help screen for CNS metastasis of lymphomas and carcinomas, regardless of pathological diagnosis. In particular, high CSF progranulin level might be a novel indicator for CNS lymphoma. While several potential diagnostic and prognostic markers for CNS lymphoma have previously been reported (Aviles et al. 1991; Hansen et al. 1992; Lee et al. 2005; Roy et al. 2008; Baraniskin et al. 2011; Wei et al. 2015b; Yu et al. 2016; Viaccoz et al. 2015; Strehlow et al. 2016; Rubenstein et al. 2013; Fischer et al. 2009; Ahluwalia et al. 2012), there is presently no reliable biomarker with high sensitivity and specificity for diagnosing CNS lymphoma. For diagnosis CNS lymphoma, it is not uncommon to perform brain biopsies, which are invasive and, in some cases, histologically inconclusive. CSF progranulin can be easily and inexpensively quantified by EIA. Further studies are needed to clarify whether CSF progranulin levels can indeed be used as a diagnostic biomarker of CNS lymphoma.

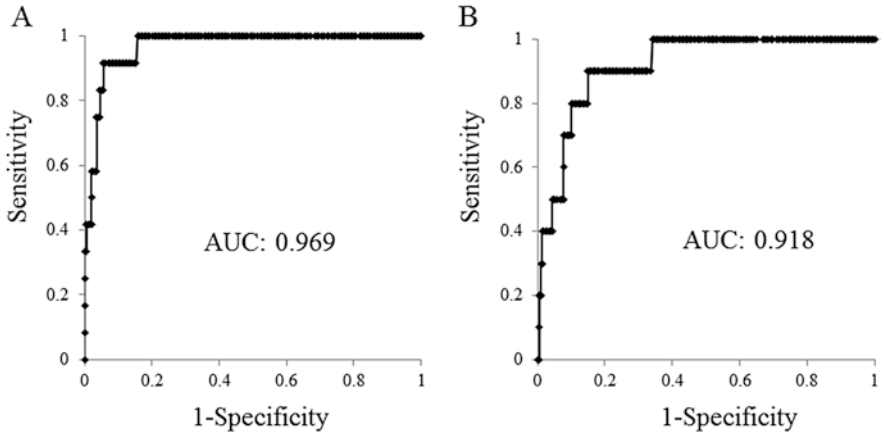

Fig. 3 Receiver operator characteristic (ROC) curve analysis of progranulin levels in cerebrospinal fluid (CSF PGRN) of (**a**) lymphoma with central nervous system (CNS) involvement (CNS+ lymphoma); and (**b**) carcinoma with CNS involvement (CNS+ carcinoma). ROC curve analyses of CSF PGRN levels could distinguish with high sensitivity and specificity, patients with CNS+ lymphoma from those without CNS involvement (CNS− lymphoma) or non-cancer neurological diseases. Similarly, CSF PGRN levels could distinguish with high sensitivity and specificity, patients with CNS+ carcinoma from those without CNS involvement (CNS− carcinoma) or non-cancer neurological diseases. AUC, area under the curve

It is unclear why CSF progranulin levels in patients with CNS metastasis of lymphomas and carcinomas are elevated. Previous immunohistochemical analysis of lymphoid malignancies in patients with diffuse large B cell lymphoma (the most common type of CNS lymphoma) showed progranulin expression in lymphoma cells and in tumor-associated activated macrophage cells (TAMs) surrounding these cells (Yamamoto et al. 2017). We speculate that increased CSF progranulin levels in patients with CNS metastasis of lymphomas and carcinomas is caused by the secretion of progranulin from tumor cells and TAMs in the CNS.

Progranulin as a Biomarker of Autoimmune Neurological Disorders

There is emerging evidence that progranulin may also be associated with various autoimmune diseases, including RA, Sjögren's syndrome, SLE, and systemic sclerosis (Jian et al. 2018). Progranulin has been shown to have therapeutic effectiveness in inflammatory arthritis by functioning as an endogenous antagonist of TNFα signaling by competitively binding to TNFR (Liu and Bosch 2012; Jian et al. 2013b; Tang et al. 2011). It was also reported that progranulin exerts its anti-inflammatory action through multiple pathways, including induction of regulatory T cell differentiation and IL-10 expression, and by inhibiting chemokine release from

macrophages (Jian et al. 2018). Serum progranulin levels are significantly higher in RA patients compared with age-matched healthy controls (Yamamoto et al. 2014). Moreover, circulating progranulin in RA patients is related to TNFα and soluble TNFR2 concentrations, and the progranulin/TNFα ratio correlates with disease stage in RA patients. High progranulin levels are also detected in serum samples from SLE patients (Tanaka et al. 2012), and serum progranulin levels are significantly associated with clinical symptoms and laboratory parameters in SLE, which are in turn related to disease activity. Importantly, serum progranulin levels are significantly decreased after successful treatment of SLE. Collectively, these observations suggest that the measurement of serum progranulin may be a useful approach for monitoring disease activity in patients with RA and SLE.

There are several reports describing the association between progranulin and CNS autoimmune neurological disorders, including multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD) (Fenoglio et al. 2010; De Riz et al. 2010; Vercellino et al. 2011, 2016; Kimura et al. 2017). Indeed, progranulin was recently reported to be strongly expressed in the brains of patients with MS, specifically, in macrophages/microglia in active lesions and in activated microglia in normal-appearing white matter (Vercellino et al. 2011). Comparison of progranulin levels in the CSF of MS patients, non-inflammatory controls and inflammatory controls revealed significantly higher progranulin concentrations during MS relapse and in patients with progressive MS compared with MS patients in remission and non-inflammatory controls. This suggests that CSF progranulin levels may be a promising marker for active MS, although one report showed unaltered CSF progranulin levels in MS patients compared with controls (De Riz et al. 2010). Previously, we compared CSF progranulin levels in 17 patients with relapsing-remitting type (RR)-MS and 20 patients with non-inflammatory neurological disorders. CSF progranulin levels were significantly higher in RR-MS patients during relapses compared with non-inflammatory controls (migraine and psychosomatic disorders) (Fig. 4). A recent study found that *GRN* polymorphisms influence the progression of disability and relapse recovery in MS, which may be related to circulating progranulin levels (Vercellino et al. 2016). It was suggested that the increased progranulin expression by microglia and macrophages in MS brain tissue might play a role in neuronal and axonal protection during brain inflammation.

NMOSD is an inflammatory disorder of the CNS that was previously thought to be a clinical subtype of MS, but more recently has been shown to be a distinct clinical and pathophysiologic entity (Katz 2016). Discovery of a disease-specific serum autoantibody against aquaporin-4 (AQP4), which is a water channel protein abundant in astrocyte foot processes surrounding brain capillaries, increased our understanding of this diverse spectrum of disorders (Lennon et al. 2005). We previously reported that CSF progranulin levels are significantly higher in NMOSD patients compared with RR-MS patients and non-inflammatory controls (Fig. 4) (Kimura et al. 2017). The elevated CSF progranulin levels correlated with CSF IL-6 levels, CSF cell count, CSF protein levels, and were related to total spinal cord lesion length in NMOSD patients. There are several additional reports showing that CSF protein levels, CSF IL-6 levels and total spinal cord lesion length during the acute phase correlate with

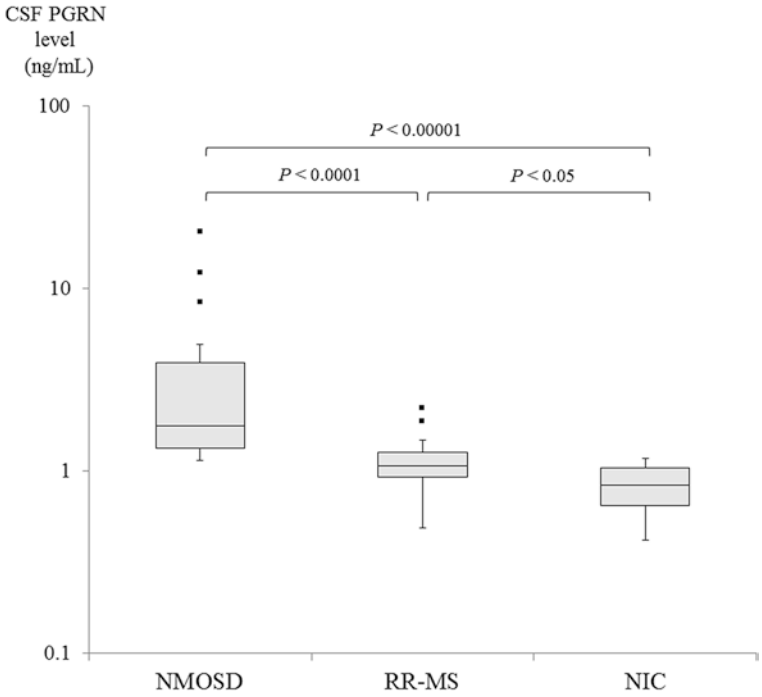

Fig. 4 Progranulin levels in cerebrospinal fluid (CSF PGRN) during the acute phase in 15 neuromyelitis optica spectrum disorder (NMOSD) patients, 17 relapsing-remitting type multiple sclerosis (RR-MS) patients, and 20 non-inflammatory controls (NIC) (4 migraine and 16 psychosomatic disorder). CSF PGRN levels were significantly higher in patients with NMOSD compared with patients with RR-MS and NIC. Similarly, CSF PGRN levels were significantly higher in patients with RR-MS compared with NIC

disease severity in NMOSD patients (Içöz et al. 2010; Jarius et al. 2011; Murchison et al. 2015). Therefore, CSF progranulin levels during the acute phase may reflect NMOSD disease severity. Moreover, CSF progranulin levels during the acute phase also correlate with improvements in expanded disability status scale (EDSS) score, which is a method for quantifying disability in MS and NMOSD patients. These findings suggest that the anti-inflammatory and neurotrophic effects of progranulin may impact recovery from relapse in NMOSD. Therefore, CSF progranulin level is a potential biomarker of disease severity and prognosis in NMOSD.

Conclusion

Progranulin levels are altered in various CNS diseases. Decreased progranulin indicates the presence of *GRN* mutations, and circulating progranulin is a useful biomarker for the rapid and inexpensive large-scale screening of *GRN* mutation carriers

in FTLD, which may be initially clinically diagnosed as another neurodegenerative disease, such as Alzheimer's or motor neuron disease. An upregulation of progranulin in the CNS is observed in various malignancies, including glioma, CNS lymphoma, carcinomatous meningitis, and brain metastasis. Hence, CSF progranulin levels could be used as a marker for monitoring CNS metastasis of lymphomas and carcinomas regardless of tumor type, which is often hard to diagnose clinically. Increased CSF progranulin levels are also observed in the acute phase of autoimmune CNS diseases such as MS and NMOSD. In the CNS, progranulin produced by microglia and macrophages might play a role in neuronal and axonal protection during the acute phase, and thereby affect recovery. CSF progranulin level might be a useful indicator of prognosis after relapse in MS and NMOSD. In addition to being a potential biomarker of CNS disease, progranulin may also hold promise as a neurotherapeutic agent.

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Progranulin and Frontotemporal Lobar Degeneration

Masato Hosokawa and Tetsuaki Arai

Abstract Granulin (*GRN*) mutations were first found in frontotemporal dementia (FTD) patients with ubiquitin-positive, tau-negative inclusions in 2006. These inclusions were also found to contain a TAR-DNA binding protein of 43 kDa (TDP-43). PGRN protein itself is not a component of ubiquitin-positive inclusion bodies. Since then, more than 190 *GRN* mutations have been reported, including substitutions, insertions, and deletions. The common pathological mechanism observed with these mutations was proposed to arise from haploinsufficiency; the amount of functional PGRN was reduced to half of the normal level. In fact, *GRN* mutation carriers showed significantly reduced expression levels of PGRN in plasma and serum. Immunohistochemical analyses of phosphorylated TDP-43 revealed that cases of FTLD-TDP with a *GRN* mutation invariably display type A pathology, which is characterized by numerous short dystrophic neurites (DNs) and crescentic or oval shaped neuronal cytoplasmic inclusions (NCIs). *GRN* mutations were initially found in FTD patients with tau-negative, TDP-43-positive inclusions, however, recent findings suggested that different clinical phenotypes may be seen in the same *GRN* mutation carriers and additional tau or α-synuclein accumulation may be observed.

Keywords Frontotemporal dementia (FTD) · Frontotemporal lobar degeneration (FTLD) · *GRN* mutation · Nonsense-mediated dacay

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Introduction

Frontotemporal lobar degeneration (FTLD) is a collective term for a disease group characterized by progressive neurodegeneration limited to frontal and temporal lobes. FTLD is clinically divided into three types: frontotemporal dementia (FTD), semantic dementia (SD) and progressive nonfluent aphasia (PNFA) (Neary et al. 1998). This classification is based on clinical manifestations that reflect differences in the degenerative brain region. They do not reflect specific neuropathological characteristics. FTLD can be subdivided into three neuropathological groups, depending on the presence of inclusion bodies or a certain protein component (McKhann et al. 2001). The first group, exhibiting "tauopathy", has tau-positive inclusion bodies. This group includes Pick's disease, corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). The second group might be called FTLD-U since it is similar to FTLD but has ubiquitin-positive tau-negative neurocytoplasmic inclusions (Mackenzie et al. 2006a). FTLD is divided into two types, FTLD with motor neuron disease: (FTLD-MND) and FTLD with MND type inclusions but without MND. The third group consist of FTLD without tau- or ubiquitin-positive inclusions, and this group has been considered a dementia lacking distinctive histology (DLDH). However, most cases of the third group consist of FTLD-U with inclusions which are identified using high-sensitivity ubiquitin immunostaining. Rare cases with tau-negative, cytoplasmic and nuclear ubiquitin-positive inclusions have also been found (Mackenzie et al. 2006c).

Some 35–50% of FTLD patients have a family history of dementia and the causative gene loci have been identified on chromosomes 3, 9 and 17. Microtubule-associated protein tau (tau, MAPT), valosin-containing protein (VCP) and charged multivesicular body protein 2b (CHMP2B) have been identified as causative of FTLD. The identification of the tau gene mutation on chromosome 17q21 reminds us of the importance of tau in neurodegenerative disease research (Hutton et al. 1998). However, a considerable number of familial FTLD-U cases linked on chromosome 17q21 with tau-negative, cytoplasmic and nuclear ubiquitin-positive inclusions have been found.

In 2006, Cruts and Baker identified a granulin (*GRN*) mutation in FTLD-U patients (Baker et al. 2006; Cruts et al. 2006). Since then, more than 190 *GRN* mutations have been reported including substitutions, insertions and deletions (Tables 1, 2 and 3 and Alzheimer Disease & Frontotemporal Dementia Mutation Database, http://www.molgen.ua.ac.be/FTDMutations/) (Cruts et al. 2012). The common pathological mechanism in these mutations was proposed to arise from haploinsufficiency. Symptoms of haploinsufficiency appear after inactivation of one allele of the causative gene in a dominantly-inherited disease (Wilkie 1994). With *GRN* mutation, a mutated form of mRNA is degraded by nonsense-mediated decay (NMD) which is likely to create a null (no expression) allele. It is thought that the functional form of the PGRN protein decreases with disease onset.

Table 1 *GRN* mutations (**pathogenic**)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
1	delGRN[DR184]	Genomic deletion of 69.1 to 74.3 kb containing *GRN, RUNDC3A and SLC25A39*		Complete gene deletion	Complete protein	FTD	71.0	74.0	1	Gijselinck et al. (2008)
2	IVS1+3A>T	g.-3828A>T		IVS1	Complete protein	FTD	58.0	n/a	1	Le Ber et al. (2007) and Brouwers et al. (2007)
3	IVS1+5G>C	g.-3826G>C		IVS1	Complete protein	FTD/PD	61.4	68.7	11	Cruts et al. (2006)
4	delGRN[French]	g.-95_3490del		IVS1-IVS12	Complete protein	FTD/PD	72.0	71.8	1	Rovelet-Lecrux et al. (2008)
5	delGRN			EX1-IVS1	Complete protein	FTLD	47.0	n/a	1	Clot et al. (2014)
6	delGRN		c.-7-1121_159delinsGATCA	IVS1-EX3	Complete protein	FTLD	58.0	65.0	1	Clot et al. (2014)
7	Met1?	g.1A>G	c.1A>G	EX2	Signal peptide	FTD/PPA	55.0	55.0	1	Le Ber et al. (2008) and Gomez-Tortosa et al. (2013)
8	Met1?	g.1A>C	c.1A>C	EX2	Signal peptide	FTLD/AD	n/a	n/a	n/a	Hosokawa et al. (2017)
9	Met1?	g.2T>C	c.2T>C	EX2	Signal peptide	FTD	51.0	n/a	2	Baker et al. (2006)
10	Met1?	g.3G>A	c.3G>A	EX2	Signal peptide	FTD	62.0	n/a	1	Cruts et al. (2006)
11	Trp2X	g.6G>A	c.6G>A	EX2	Signal peptide	FTD	61.0	n/a	1	Mendez (2018)

(continued)

Table 1 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
12	Ala9Asp	g.26C>A	c.26C>A	EX2	Signal peptide	FTD/PPA	56.2	63.4	6	Mukherjee et al. (2006), Gass et al. (2006), Spina et al. (2007), Ghetti et al. (2008), Mukherjee et al. (2008), Spina et al. (2008), Kelley et al. (2009), and Yu et al. (2010)
13	Asp22ArgfsX43	g.63_64insC	c.63_64insC	EX2	ParaGran	FTD	64.2	73.0	2	Gass et al. (2006) and Pietroboni et al. (2011)
14	Cys31LeufsX34	g.90_91insCTGC	c.90_91insCTGC	EX2	ParaGran	FTD	57.9	64.5	6	Baker et al. (2006), Gass et al. (2006), Beck et al. (2008), Rohrer et al. (2008, 2009, 2010a, b), and Yu et al. (2010)
15	Gly35GlufsX19	g.102delC	c.102delC	EX2	ParaGran	FTD/PPA	68.5	58.2	3	Gass et al. (2006), Chiang et al. (2008), and Skoglund et al. (2009)
16	IVS2+1G>A	g.139G>A	c.-7_138del	IVS2	Complete protein	FTD	58.1	68.8	2	Gass et al. (2006), Boeve et al. (2006), Pickering-Brown et al. (2006), and Kelley et al. (2009)

17	Thr52HisfsX2	g.277delA	c.154delA	EX3	InterParaG	FTD/AD	66.8	81.5	5	Gass et al. (2006), Kelley et al. (2009, 2010), and Lindquist et al. (2009)
18	Gly79AspfsX39	g.357_358delAG	c.234_235delAG	EX3	GranG	FTD	56.0	67.0	2	Gass et al. (2006)
19	Ser82ValfsX174	g.366delC	c.243delC	EX3	GranG	FTD	61.2	70.7	1	Bronner et al. (2007)
20	Phe86SerfsX170	g.378delC	c.255delC	EX3	GranG	FTD	n/a	n/a	1	Gijselinck et al. (2008)
21	Ala89ValfsX41 (IVS3+2T>C)	g.389T>C	c.0 (C.264_265ins34)	IVS3	GranG	FTD	60.0	71.0	2	Gijselinck et al. (2008), Yu et al. (2010) and Gomez-Tortosa et al. (2013)
22	IVS3-2delA	g.385delA		IVS3	GranG	PPA	63.0	n/a	1	Cioffi et al. (2016)
23	Pro100HisfsX156	g.537delC	c.299delC	EX4	GranG	FTD	60.0	n/a	1	Yu et al. (2010)
24	Cys105TrpfsX13	g.552dup	c.314dup	EX4	GranG	CBD	n/a	n/a	1	Dopper et al. (2011)
25	Arg110X	g.566C>T	c.328C>T	EX4	GranG	FTD	54.0	n/a	3	Van Deerlin et al. (2007), Le Ber et al. (2008), and Yu et al. (2010)
26	Ser116X	g.585C>A	c.347C>A	EX4	interGF	FTD	57	n/a	1	Yu et al. (2010)
27	Asn118PhefsX4	g.1064delG	c.350_462del	EX5	interGF	PPA	69.0	n/a	2	Le Ber et al. (2008) and Gijselinck et al. (2008)
28	Val121TrpfsX135	g.1075delG	c.361delG	EX5	InterGF	FTD	54.5	63.0	1	Le Ber et al. (2007, 2008)

(continued)

Table 1 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
29	Gln125X	g.1087C>T	c.373C>T	EX5	GranF	FTD	64.9	70.0	1	Baker et al. (2006), Cruts et al. (2006), and Bronner et al. (2007)
30	Pro127ArgfsX2	g.1094_1095delCT	c.380_381delCT	EX5	GranF	FTD	55.5	n/a	2	Cruts et al. (2006) and Le Ber et al. (2008)
31	Gln130SerfsX125	g.1098_1101delTAGT	c.384_387delTAGT	EX5	GranF	FTD	45.0	49.0	1	Le Ber et al. (2007, 2008)
32	Gln130SerfsX125	g.1102_1105delCAGT	c.388_391delCAGT	EX5	GranF	FTD	64.2	68.0	8	Baker et al. (2006), Gass et al. (2006), Beck et al. (2008), Finch et al. (2009), Carecchio et al. (2009), Yu et al. (2010), and Rohrer et al. (2010a, b)
33	Cys149LeufsX10	g.1159_1160delTG	c.445_446delTG	EX5	GranF	FTLD/AD	62.6	n/a	11	Calvi et al. (2015) and Piaceri et al. (2018)
34	Ala155TrpfsX56	g.1277G>A	c.0 (c.463_598del)	IVS5	GranF	FTD	63.3	60.0	2	Gass et al. (2006) and Le Ber et al. (2008)
35	Cys157LysfsX97	g.1283_1289delCTGCTGT	c.468_474delCTGCTGT	EX6	GranF	FTD/CBS	52.0	61.0	1	Le Ber et al. (2007, 2008) and Coppola et al. (2012)

36	Arg161GlyfsX36	g.1296_1297delAG	C.481_482delAG	EX6	GranF	PPA	51.0	n/a	1	Gazzina et al. (2017)
37	Arg198GlyfsX19	g.1407_1408delAG	c.592_593delAG	EX6	InterFB	AD	60.5	n/a	2	Finch et al. (2009) and Yu et al. (2010)
38	Ala199Val	g.1411C>T	c.596C>T	EX6	InterFB	FTLD	n/a	n/a	1	Luzzi et al. (2017)
39	IVS6+2_5delTGAG			IVS6	InterFB	FTD	n/a	58.5	n/a	Mao et al. (2017)
40	IVS6+5_8delGTGA			IVS6	InterFB	FTD/AD	n/a	n/a	1	Marcon et al. (2011)
41	Ser203ValfsX15	g.1531_1532insC	c.603_604insC	EX7	InterFB	FTD	n/a	n/a	1	Beck et al. (2008) and Rohrer et al. (2010a, b)
42	Ser226TrpfsX28	g.1603_1604delCA	c.675_676delCA	EX7	GranB	FTD/PPA	58.0	63.0	7	Gass et al. (2006), Van Deerlin et al. (2007), Davion et al. (2007), Coppola et al. (2008), Yu et al. (2010), and Kim et al. (2016)
43	Tyr229X	g.1615T>A	c.687T>A	EX7	GranB	FTLD	60.0	70.5	1	Kuuluvainen et al. (2017)
44	Val200GlyfsX18 (IVS7+1G>A)	g.1637G>A	c.0 (c.599_708del)	IVS7	InterFB	FTD/CBS	60.0	64.3	3	Masellis et al. (2006) and Le Ber et al. (2007, 2008)
45	Val200GlyfsX18 (IVS7+1G>C)	g.1637G>C	c.0 (c.599_708del)	IVS7	InterFB	FTD	55.0	61.0	1	Gass et al. (2006)
46			c.708+6_+9delTGAG	IVS7	InterFB	FTD/AD	60.1	70.2	n/a	Bit-Ivan et al. (2014)
47			c.709-2A>T	IVS7	InterFB	FTD/PPA	67.0	74.6	1	Sassi et al. (2016)

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Table 1 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
48	Ala237TrpfsX6	g.1871A>G	c.0 (c.709_835del)	IVS7	GranB	FTD/PPA	58.4	67.0	9	Behrens et al. (2007), Spina et al. (2007), Leverenz et al. (2007), Davion et al. (2007), Ghetti et al. (2008), Mukherjee et al. (2008), Yu et al. (2010), and Kim et al. (2016)
49	Ala237TrpfsX4	g.1872G>A	c.0 (c.709_835del)	IVS7	GranB	FTD/ CBS/ MND	59.0	63.0	13	López de Munain et al. (2008) and Moreno et al. (2009)
50	Ala237TrpfsX4	g.1999_2000insCTGA	c.0 (c.709_835del)	IVS7	GranB	FTD	67.0	n/a	1	Cruts et al. (2006)
51	Cys253X	g.1923_1924delTG	c.759_760delTG	EX8	GranB	FTD	59.5	65.0	2	Gass et al. (2006) and Le Ber et al. (2008)
52	Gln257ProfsX27	g.1933_1934insCC	c.769_770insCC	EX8	GranB	AD	54.8	n/a	3	Jin et al. (2012), Pires et al. (2013), and Almeida et al. (2014)
53	Lys259X	g.1939A>T	c.775A>T	EX8	GranB	FTD	54.5	n/a	1	Schlachetzki et al. (2009)

54	Leu271LeufsX10	g.1975_1978delCTCA	c.811_814delCTCA	EX8	InterBA	FTLD/CBS	63.8	69.4	2	Benussi et al. (2008)
55	Thr272SerfsX10	g.1977_1980delCACT	c.813_816delCACT	EX8	InterBA	FTD/PSP	60.1	71.0	35	Le Ber et al. (2008), Borroni et al. (2008), Benussi et al. (2008), Tremolizzo et al. (2009), Carecchio et al. (2009), Yu et al. (2010), and Arosio et al. (2013)
56	Thr278SerfsX7	g.1997_1998delCA	c.0 (c.829_834)	IVS8	InterBA	FTLD	60.5	74.3	1	Rossi et al. (2011)
57	Val279GlyfsX5 (IVS8-1G>C)	g.2198G>C	c.0 (c.836_933del)	IVS8	InterBA	FTD/CBS	56.0	n/a	2	Gass et al. (2006) and Coppola et al. (2008)
58	Asp285GlufsX3	g.2211_2217dupAATGTGA	c.848_854dupAATGTGA	EX9	GranA	FTLD-U	53.0	n/a	1	Yu et al. (2010)
59	Tyr294X	g.2245T>G	c.882T>G	EX9	GranA	FTD	50.0	n/a	1	Alzheimer Disease & Frontotemporal Dementia Mutation Database
60	Gln300X	g.2261C>T	c.898C>T	EX9	GranA	FTD	n/a	n/a	1	Beck et al. (2008) and Rohrer et al. (2009)
61	Ser301CysfsX61	g.2264_2265insGT	c.901_902insGT	EX9	GranA	FTD/CBS	52.7	58.0	3	Guerreiro et al. (2008) and Almeida et al. (2014)
62	Ala303GlyfsX14	g.2270_2271insG	c.907_908insG	EX9	GranA	FTD	n/a	n/a	1	Gijselinck et al. (2008)

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Table 1 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
63	Ala303AlafsX57	g.2271delC		EX9	GranA	FTLD	n/a	n/a	n/a	Gomez-Tortosa et al. (2013)
64	Trp304GlyfsX57	g.2272delC	c.909delC	EX9	GranA	FTD/PD	59.5	74.0	2	Lladó et al. (2007) and Almeida et al. (2014)
65	Trp304LeufsX58	g.2273_2274insTG	c.910_911insTG	EX9	GranA	FTD	58.0	65.0	2	Gass et al. (2006), Kelley et al. (2009), and Kim et al. (2016)
66	Trp304X	g.2274G>A	c.911G>A	EX9	GranA	FTD	61.7	65.0	4	Gass et al. (2006), Van Deerlin et al. (2007), and Yu et al. (2010)
67	Trp304Cys	g.2275G>C	c.912G>C	EX9	GranA	FTD/AD	56.7	n/a	1	Piaceri et al. (2018)
68	Val279GlyfsX5	g.2297G>A	c.0 (c.836_933del)	IVS9	GranA	FTD	61.0	n/a	1	Baker et al. (2006) and Gass et al. (2006)
69	Cys314X	g.2394C>A	c.942C>A	EX10	GranA	FTD	70.5	78.0	2	Le Ber et al. (2007, 2008)
70	Gly333ValfsX28	g.2450delG	c.998delG	EX10	GranA	PPA	62.0	72.0	1	Gass et al. (2006), Mesulam et al. (2007) and Kelley et al. (2009)
71	Gln337X	g.2461C>T	c.1009C>T	EX10	InterAC	FTD	62.0	n/a	2	Van Deerlin et al. (2007) and Yu et al. (2010)

72	Gly338GlyfsX22	n/a	n/a	EX10	InterAC	FTLD	n/a	n/a	n/a	Cupidi et al. (2009, abst)
73	His340ThrfsX21	g.2466delG	c.1014delG	EX10	InterAC	FTD	n/a	n/a	1	Benussi et al. (2008)
74	Gln341X	g.2473C>T	c.1021C>T	EX10	InterAC	PPA	63.0	72.0	1	Benussi et al. (2008)
75	Pro357HisfsX4	g.2522delC	c.1070delC	EX10	InterAC	FTD	44.0	51.0	1	López de Munain et al. (2008)
76	Gln358X	g.2524C>T	c.1072C>T	EX10	InterAC	FTD	n/a	n/a	1	Spina et al. (2008)
77	Cys366fsX1	g.2547_2548delCT	c.1095_1096delCT	EX10	GranC	FTD	74.0	85.0	1	Le Ber et al. (2007, 2008)
78	Pro373ArgfsX37	g.2570_2571delCCinsG	c.1118_1119delCCinsG	EX10	GranC	PPA	n/a	n/a	n/a	Hosaka et al. (2017)
79	Thr382GlnfsX32	g.2596_2597insA	c.1144_1145insA	EX10	GranC	FTD	63.4	70.0	1	Bruni et al. (2007) Frangipane et al. (2008)
80	Thr382SerfsX30	g.2597delC	c.1145delC	EX10	GranC	FTD/CBS	54.0	n/a	1	Baker et al. (2006), Gass et al. (2006), and Kelley et al. (2009)
81	Trp386X	g.2609G>A	c.1157G>A	EX10	GranC	FTD	62.7	71.0	3	Baker et al. (2006), Gass et al. (2006), Lindquist et al. (2009), and Yu et al. (2010)
82	Gly387fsX25	n/a	n/a	EX10	GranC	PD	51.0	n/a	1	Carecchio et al. (2014)
83	Ala394LeufsX18	g.2632delG	c.1179delG	IVS10	GranC	FTD	38.0	n/a	2	Almeida et al. (2014)

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Table 1 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
84	Glu316_Cys397del	g.2633T>C	c.939_1184del	IVS10	GranA; InterAC; GranC	FTD	59.0	n/a	1	Yu et al. (2010)
85	Asp399Val	n/a	n/a	EX11	GranC	FTLD	n/a	n/a	n/a	Cupidi et al. (2009, abst)
86	Gln401X	g.2872C>T	c.1201C>T	EX11	GranC	FTD	58.3	60.5	2	Le Ber et al. (2007, 2008)
87	Thr409Met	g.2897C>T	c.1226G>A	EX11	GranC	PPA	n/a	n/a	1	Cerami et al. (2013)
88	Val411SerfsX2	g.2902_2903delGT	c.1231_1232delGT	EX11	GranC	FTD	66.0	n/a	1	Bronner et al. (2007)
89	Ala412fsX1	g.2903_2904insGT	c.1232_1233insGT	EX11	GranC	FTD	57.5	68.0	2	Le Ber et al. (2007, 2008)
90	Gln415X	g.2914C>T	c.1243C>T	EX11	GranC	FTD	n/a	n/a	4	Pickering-Brown et al. (2008)
91	Arg418X	g.2923C>T	c.1252C>T	EX11	InterCD	FTD	56.3	60.3	5	Baker et al. (2006), Gass et al. (2006), Van Deerlin et al. (2007), Schlachetzki et al. (2009), and Yu et al. (2010)
92	Asp441HisfsX4	g.2988_2989delCA	c.1317_1318delCA	EX11	InterCD	CBS	55.0	n/a	1	Yu et al. (2010)
93	Cys466LeufsX46	g.3066_3067insC	c.1395_1396insC	EX11	GranD	FTD	52.0	56.0	1	Gass et al. (2006) and Kelley et al. (2009)

94	Gln468X	g.3073C>T	c.1402C>T	EX11	GranD	FTD/PPA	60.3	68.8	2	Baker et al. (2006) and Yu et al. (2010)
95	Ala472ValfsX10 (IVS11-15_ EX12+177del)	g.3162_3354del	c.1413_1414ins92; 1644_1645ins89	EX12	GranD; InterDE; GranE	FTD	64.5	86.0	3	Pickering-Brown et al. (2006), Finch et al. (2009), and Yu et al. (2010)
96	Ala472_Gln548del	g.3175A>G	c.1415_1645del	IVS11	GranD; InterDE; GranE	FTD	39.0	n/a	1	Yu et al. (2010)
97	Cys474LeufsX37	g.3183_3184delTG	c.1420_1421delTG	EX12	GranD	FTD	n/a	n/a	1	Spina et al. (2008)
98	Thr487Ile	g.3223C>T	c.1460C>T	EX12	GranD	PD	n/a	n/a	n/a	Chang et al. (2018)
99	Arg493X	g.3240C>T	c.1477C>T	EX12	GranD	FTLD	57.0	63.6	44	Huey et al. (2006), Gass et al. (2006), Pickering-Brown et al. (2006), Mesulam et al. (2007), Spina et al. (2007), Van Deerlin et al. (2007), Davion et al. (2007), Rademakers et al. (2007), Ghetti et al. (2008), Spina et al. (2008), Kelley et al. (2009), Rohrer et al. (2010a, b), Yu et al. (2010), and Kim et al. (2016)

(continued)

Table 1 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
100	Cys495Cys	g.3248C>T	c.1485C>T	EX12	GranD	FTD	n/a	n/a	n/a	van der Zee et al. (2007)
101	Glu498AspfsX12	g.3257_3261delAGTGG	c.1494_1498delAGTGG	EX12	InterDE	FTD	57.2	n/a	4	Beck et al. (2008), Le Ber et al. (2008), and Rohrer et al. (2010a, b)
102	Gln503X	g.3270C>T	c.1507C>T	EX12	InterDE	FTD	75.0	n/a	1	Aswathy et al. (2016)
103	Val516GlyfsX31	n/a	n/a	EX12	InterDE	FTLD	n/a	n/a	n/a	Cupidi et al. (2009, Abst)
104	Cys521Tyr	g.3325G>A	c.1562G>A	EX12	GranE	FTD	60.5	71.8	1	Cruchaga et al. (2009)
105	Arg535X	g.3366C>T	c.1603C>T	EX12	GranE	AD	72.0	n/a	1	Brouwers et al. (2007)

n/a not applicable, *EX* exon, *IVS* intervening sequence, *Gran* Granulin. Phenotype: *AD* Alzheimer's disease, *CBS* corticobasal syndrome, *FTD* frontotemporal dementia, *FTLD* frontotemporal lobar degeneration, *FTLD-U* frontotemporal lobar degeneration with ubiquitin-positive inclusions, *MND* motor neuron disease, *PD* Parkinson's disease, *PPA* primary progressive aphasia, *PSP* progressive supranuclear palsy

Table 2 *GRN* mutations (**pathogenic nature unknown**)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
1	Val5Leu	g.13G>C	c.13G>C	EX2	Signal peptide	FTD-MND	63.0	65.0	1	López de Munain et al. (2008)
2	Trp7Arg	g.19T>C	c.19T>C	EX2	Signal peptide	FTD	51.0	n/a	1	Le Ber et al. (2008)
3	Asp33Glu	g.99C>A	c.99C>A	EX2	ParaGran	AD/PD	68.5	85.0	2	Brouwers et al. (2008) and Nuytemans et al. (2008)
4	Pro34Ser	g.100C>T	c.100C>T	EX2	ParaGran	FTD	n/a	n/a	n/a	Almeida et al. (2014)
5	Pro34Pro	g.102C>T	c.102C>T	EX2	ParaGran	FTD	n/a	n/a	1	van der Zee et al. (2007) and Le Ber et al. (2007)
6	Gly35Arg	g.103G>A	c.103G>A	EX2	ParaGran	AD	n/a	n/a	1	Galimberti et al. (2008) and Cortini et al. (2008)
7	Leu53Pro	g.281T>C	c.158T>C	EX3	InterParaG	FTD	n/a	n/a	n/a	Skoglund et al. (2007)
8	Gly70Ser	g.331G>A	c.208G>A	EX3	GranG	FTD	55.0	n/a	1	Yu et al. (2010)
9	Val77Ile	g.352G>A	c.229G>A	EX3	GranG	At risk	n/a	n/a	1	Yu et al. (2010)
10	Glu88Glu	g.387G>A	c.264G>A	EX3	GranG	FTLD	n/a	n/a	n/a	Gass et al. (2006)
11	Cys105Arg	g.551T>C	c.313T>C	EX4	GranG	FTD	39.0	n/a	1	Gass et al. (2006) and Yu et al. (2010)
12	Cys105Tyr	g.552G>A	c.314G>A	EX4	GranG	FTD	61.5	n/a	1	Karch et al. (2016)
13	Arg110Gln	g.567G>A	c.329G>A	EX4	GranG	ALS	n/a	n/a	n/a	Sleegers et al. (2008)
14	Ile124Thr	g.1085T>C	c.371T>C	EX5	GranF	ALS	n/a	n/a	n/a	Sleegers et al. (2008)
15	Thr138Thr	g.1128G>A	c.414G>A	EX5	GranF	ALS	n/a	n/a	n/a	Sleegers et al. (2008)
16	Cys139Arg	g.1129T>C	c.415T>C	EX5	GranF	FTD/PPA/AD	72.3	89.0	3	Brouwers et al. (2008), Finch et al. (2009), Bernardi et al. (2009), and Redaelli et al. (2018)

(continued)

Table 2 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
17	Gly168Ser	g.1317G>A	c.502G>A	EX6	GranF	FTLD/ MND	n/a	n/a	1	Pickering-Brown et al. (2008)
18	Arg177His	g.1345G>A	c.530G>A	EX6	GranF	FTD	70.0	78.0	1	López de Munain et al. (2008), Schymick et al. (2007), Guerreiro et al. (2008), Guerreiro et al. (2010), and Yu et al. (2010)
19	Ala199Val	g.1411C>T	c.596C>T	EX6	interFB	CBS	62.0	n/a	2	Beck et al. (2008), Rohrer et al. (2010a, b), and Karch et al. (2016)
20	Arg212Trp	g.1562C>T	c.634C>T	EX7	GranB	FTD	n/a	n/a	1	Yu et al. (2010)
21	Cys222Tyr	g.1593G>A	c.665G>A	EX7	GranB	AD	n/a	n/a	n/a	Lee et al. (2014)
22	Pro233His	g.1626C>A	c.698C>A	EX7	GranB	FTLD	n/a	n/a	1	Bronner et al. (2007)
23	Asn236Asn	g.1636C>T	c.708C>T	EX7	GranB	FTLD	n/a	n/a	n/a	Gass et al. (2006)
24	IVS7-3C>G	g.1870C>G	c.709_835del	IVS7	GranB	FTD	45.0	n/a	1	Benussi et al. (2008)
25	IVS7-1G>A	g.1872G>A	c.709-1G>A	IVS7	GranB	UI	n/a	n/a	n/a	Barandiaran et al. (2012)
26	Pro248Leu	g.1907C>T	c.743C>T	EX8	GranB	FTD	n/a	n/a	n/a	van der Zee et al. (2007) and Le Ber et al. (2007)
27	Thr251Ser	g.1916C>G	c.752C>G	EX8	GranB	FTD	44.0	n/a	1	Yu et al. (2010)
28	Ser258Asn	g.1937G>A	c.773G>A	EX8	GranB	FTD	n/a	n/a	n/a	van der Zee et al. (2007) and Le Ber et al. (2007)
29	Ala276Val	g.1991C>T	c.827C>T	EX8	InterBA	Dep	50.0	n/a	1	Yu et al. (2010)
30	Glu287Asp	g.2224G>C	c.861G>C	EX9	GranA	FTLD	n/a	n/a	n/a	Gass et al. (2006)
31	Arg298His	g.2256G>A	c.893G>A	EX9	GranA	FTD	67.0	n/a	1	Yu et al. (2010) and Karch et al. (2016)
32	Ser353Asn	g.2510G>A	c.1058G>A	EX10	InterAC	FTD	65.0	n/a	1	Yu et al. (2010)
33	Pro357Arg	g.2522C>G	c.1070C>G	EX10	InterAC	FTD	62.0	n/a	1	Yu et al. (2010)
34	G387fsX25	n/a	n/a	EX10	GranC	PD	n/a	n/a	n/a	Carecchio et al. (2014)

35	Pro392Pro	g.2628A>C	c.1176A>C	EX10	GranC	ALS	n/a	n/a	1	Schymick et al. (2007)
36	Arg432Cys	g.2965C>T	c.1294C>T	EX11	InterCD	FTD	75.0	n/a	3	van der Zee et al. (2007), Le Ber et al. (2007), and Sassi et al. (2014)
37	His447His	g.3012C>T	c.1341C>T	EX11	GranD	FTD/ALS	n/a	n/a	n/a	Bronner et al. (2007), Sleegers et al. (2008), and Finch et al. (2009)
38	Pro451Leu	g.3023C>T	c.1352C>T	EX11	GranD	AD	74.0	78.0	1	Brouwers et al. (2008)
39	Leu469Phe	g.3078G>T	c.1407G>T	EX11	GranD	PA	n/a	n/a	1	Beck et al. (2008) and Rohrer et al. (2009)
40	Cys474Cys	g.3185C>T	c.1422C>T	EX12	GranD	FTD	n/a	n/a	n/a	Gass et al. (2006)
41	Val514Met	g.3303G>A	c.1540G>A	EX12	InterDE	AD/PD	71.5	82.0	n/a	Brouwers et al. (2008) and Nuytemans et al. (2008)
42	Val519Met	g.3318G>A	c.1555G>A	EX12	GranE	AD	n/a	n/a	4	Lee et al. (2014)
43	Trp541Cys	g.3386G>C	c.1623G>C	EX12	GranE	FTLD	n/a	n/a	1	Bronner et al. (2007)
44	Arg547Cys	g.3402C>T	c.1639C>T	EX12	GranE	PD	54.0	n/a	1	Wong et al. (2008, per. Comm, 2009)
45	Arg564Cys	g.3542C>T	c.1690C>T	EX13	GranE	AD	70.0	n/a	1	Brouwers et al. (2008)
46	Pro578Pro	g.3586G>A	c.1734G>A	EX13	C-Term	PPA	55.0	n/a	1	Finch et al. (2009)

n/a not applicable, *EX* exon, *IVS* intervening sequence, *Gran* Granulin. Phenotype: *ALS* amyotrophic lateral sclerosis, *AD* Alzheimer's disease, *CBS* corticobasal syndrome, *Dep* depression, *FTD* frontotemporal dementia, *FTLD* frontotemporal lobar degeneration, *FTLD-U* frontotemporal lobar degeneration with ubiquitin-positive inclusions, *MND* motor neuron disease, *PD* Parkinson's disease, *PPA* primary progressive aphasia, *PSP* progressive supranuclear palsy

Table 3 *GRN* mutations (**not pathogenic**)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
1	Leu14Leu	g.42G>A	c.42G>A	EX2	Signal peptide	FTD/CTRL	n/a	n/a	n/a	Guerreiro et al. (2008, 2010)
2	Arg19Trp	g.55C>T	c.55C>T	EX2	ParaGran	CTRL	n/a	n/a	n/a	Gass et al. (2006), Schymick et al. (2007), Guerreiro et al. (2010), and Del Bo et al. (2011)
3	Asp33Asp	g.99C>T	c.99C>T	EX2	ParaGran	CTRL	n/a	n/a	n/a	Gass et al. (2006), van der Zee et al. (2007), Schymick et al. (2007), Le Ber et al. (2007), Mukherjee et al. (2008), Sleegers et al. (2008), Cortini et al. (2008), Chiang et al. (2008), Guerreiro et al. (2010), Yu et al. (2010), and Del Bo et al. (2011)
4	Leu53Leu	g.282G>A	c.159G>A	EX3	InterParaG	FTD	55.0	n/a	n/a	Yu et al. (2010)
5	Arg55Trp	g.286A>T	c.163A>T	EX3	InterParaG	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
6	Ala69Thr	g.328G>A	c.205G>A	EX3	GranG	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
7	Thr76Thr	g.351C>T	c.228C>T	EX3	GranG	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
8	Ala89Ala	g.505C>T	c.267C>T	EX4	GranG	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
9	Gly93Gly	g.517G>A	c.279G>A	EX4	GranG	FTD/UI	n/a	n/a	n/a	Bronner et al. (2007)
10	Ser106Asn	g.555G>A	c.317G>A	EX4	GranG	UI	n/a	n/a	n/a	van der Zee et al. (2007)
11	Asp108Asp	g.562C>T	c.324C>T	EX4	GranG	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
12	Asn119del	g.1069_1071delAAC	c.355_357delAAC	EX5	InterGF	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
13	Ser120Tyr	g.1073C>A	c.359C>A	EX5	InterGF	ALS-FTD/ FTD/CTRL	63.0	63.0	n/a	Schymick et al. (2007), Guerreiro et al. (2010), and Del Bo et al. (2011)

14	Asp128Asp	g.1098T>C	c.384T>C	EX5	GranF	CTRL	n/a	n/a	n/a	Gass et al. (2006), van der Zee et al. (2007), Guerreiro et al. (2008), Sleegers et al. (2008), Tremolizzo et al. (2009), Guerreiro et al. (2010), and Del Bo et al. (2011)
15	Pro134Pro	g.1116G>A	c.402G>A	EX5	GranF	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
16	Val141Ile	g.1135G>A	c.421G>A	EX5	GranF	CTRL	n/a	n/a	n/a	Schymick et al. (2007)
17	Cys158Tyr	g.1288G>A	c.473G>A	EX6	GranF	UI	n/a	n/a	n/a	van der Zee et al. (2007)
18	Ala169Ala	g.1322C>G	c.507C>G	EX6	GranF	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
19	Thr182Met	g.1360C>T	c.545C>T	EX6	InterFB	FTD/ALS/ MSA/CTRL	52.0	n/a	n/a	Bronner et al. (2007), Schymick et al. (2007), Guerreiro et al. (2008, 2010), and Yu et al. (2010)
20	Thr182Thr	g.1361G>A	c.546G>A	EX6	InterFB	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
21	Arg212Gln	g.1563G>A	c.635G>A	EX7	GranB	UI	n/a	n/a	n/a	van der Zee et al. (2007) and Brouwers et al. (2008)
22	Thr220Ser	g.1586A>T	c.658A>T	EX7	GranB	UI	n/a	n/a	n/a	van der Zee et al. (2007)
23	Cys221Ser	g.1590G>C	c.662G>C	EX7	GranB	UI	n/a	n/a	n/a	Guerreiro et al. (2010)
24	Leu261Ile	g.1945C>A	c.781C>A	EX8	InterBA	AD/UI	n/a	n/a	n/a	van der Zee et al. (2007) and Brouwers et al. (2008)
25	Ser262Ser	g.1950C>T	c.786C>T	EX8	InterBA	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
26	Thr268Thr	g.1968G>A	c.804G>A	EX8	InterBA	ALS/UI	n/a	n/a	n/a	van der Zee et al. (2007) and Sleegers et al. (2008)
27	Ser301Ser	g.2266G>A	c.903G>A	EX9	GranA	FTD/ALS/ UI	n/a	n/a	n/a	Gass et al. (2006), van der Zee et al. (2007), Sleegers et al. (2008), and Yu et al. (2010)

(continued)

Table 3 (continued)

	Mutation (protein)	Mutation (genomic DNA)	Mutation (cDNA)	Exon	Domain	Phenotype	Mean onset ages	Mean age at death	Frequency (family)	Citations
28	Ala324Thr	g.2422G>A	c.970G>A	EX10	GranA	CTRL	n/a	n/a	n/a	Gass et al. (2006), Schymick et al. (2007), Beck et al. (2008), Pickering-Brown et al. (2008), Sleegers et al. (2008), Brouwers et al. (2008), Nuytemans et al. (2008), Finch et al. (2009), Yu et al. (2010), and Sassi et al. (2014)
29	Lys332Lys	g.2448G>A	c.996G>A	EX10	GranA	UI	n/a	n/a	n/a	van der Zee et al. (2007)
30	Asp376Asn	g.2578G>A	c.1126G>A	EX10	GranC	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
31	Ser398Leu	g.2864C>T	c.1193C>T	EX11	GranC	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
32	Thr409Thr	g.2898G>A	c.1227G>A	EX11	GranC	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
33	Gly414Val	g.2912G>T	c.1241G>T	EX11	GranC	FTLD/ CTRL	n/a	n/a	n/a	Bronner et al. (2007)
34	Arg418Gln	g.2924G>A	c.1253G>A	EX11	InterCD	CTRL	n/a	n/a	n/a	Gass et al. (2006), Bronner et al. (2007), van der Zee et al. (2007), Le Ber et al. (2007), Sleegers et al. (2008), and Yu et al. (2010)
35	Arg433Trp	g.2968C>T	c.1297C>T	EX11	InterCD	CTRL	n/a	n/a	n/a	Gass et al. (2006), Spina et al. (2007), van der Zee et al. (2007), Schymick et al. (2007), Beck et al. (2008), Brouwers et al. (2008), Nuytemans et al. (2008), Finch et al. (2009), Yu et al. (2010), and Sassi et al. (2014)
36	Arg433Gln	g.2969G>A	c.1298G>A	EX11	InterCD	FTD/CTRL	73.0	n/a	n/a	Mukherjee et al. (2008)
37	Pro458Leu	g.3044C>T	c.1373C>T	EX11	GranD	CTRL	n/a	n/a	n/a	Schymick et al. (2007)
38	Pro470Leu	g.3080C>T	c.1409C>T	EX11	GranD	UI	n/a	n/a	n/a	van der Zee et al. (2007)
39	Cys475Cys	g.3188C>T	c.1425C>T	EX12	GranD	UI	n/a	n/a	n/a	van der Zee et al. (2007)

40	Ala505Ala	g.3278C>T	c.1515C>T	EX12	InterDE	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
41	Gly515Ala	g.3307G>C	c.1544G>C	EX12	InterDE	CTRL	n/a	n/a	n/a	Gass et al. (2006), van der Zee et al. (2007), Brouwers et al. (2008), Guerreiro et al. (2010), and Yu et al. (2010)
42	Asp518Asp	g.3317C>T	c.1554C>T	EX12	GranE	ALS/CTRL	n/a	n/a	n/a	Guerreiro et al. (2010) and Del Bo et al. (2011)
43	Val550Ile	g.3500G>A	c.1648G>A	EX13	GranE	UI	n/a	n/a	n/a	van der Zee et al. (2007)
44	Arg556Cys	g.3518C>T	c.1666C>T	EX13	GranE	CTRL	n/a	n/a	n/a	Schymick et al. (2007)
45	Arg564His	g.3543G>A	c.1691G>A	EX13	GranE	CTRL	n/a	n/a	n/a	Guerreiro et al. (2010)
46	Cys565Cys	g.3547C>T	c.1695C>T	EX13	GranE	FTD/CTRL	n/a	n/a	n/a	van der Zee et al. (2007), Le Ber et al. (2007) and Guerreiro et al. (2010)
47	Ala582Thr	g.3596G>A	c.1744G>A	EX13	C-Term	CTRL	n/a	n/a	n/a	Sassi et al. (2014)

n/a not applicable, *EX* exon, *IVS* intervening sequence, *Gran* Granulin. Phenotype: *ALS* amyotrophic lateral sclerosis, *AD* Alzheimer's disease, *CTRL* control, *FTD* frontotemporal dementia, *FTLD* frontotemporal lobar degeneration, *MND* motor neuron disease, *UI* unaffected individual

A TAR-DNA binding protein of 43 kDa (TDP-43), the main component of ubiquitin-positive inclusions, was observed in FTLD and ALS patients and was identified in 2006 by Arai et al. and Neumann et al. (Arai et al. 2006; Neumann et al. 2006). The tau-negative, ubiquitin-positive inclusions that were seen in *GRN* mutation brains were also identified as containing TDP-43.

GRN Mutations and Pathological Mechanisms

Baker and colleagues examined more than 80 candidate genes within the 3.53-cM (6.19-Mb) critical region clarified by haplotype analysis of Canadian tau-negative FTD families (Baker et al. 2006). They identified an insertion mutation of four base pairs (CTGC) in exon 1 of the *GRN* gene (g.90_91insCTGC) [g: genomic DNA, ins: insertion]. The numbering is relative to the reverse complement of GenBank accession number AC003043.1, starting at adenine (A) of Met 1. This mutation causes a frame shift at codon 31 that induces a premature termination codon after a read through of 34 amino acids (p.Cys31LeufsX34) [p: protein, fs: frame shifts, X: termination codon]. The p.Cys31LeufsX34 mutation was absent in 550 North American control individuals. They sequenced the *GRN* gene in affected families in Canada, the USA, UK, Netherlands and Scandinavia and identified an additional eight *GRN* mutations in nine families. These mutations were as follows: four nonsense mutations: g.1087C>T (p.Gln125X), g.2609G>A (p.Trp386X), g.2923C>T (p.Arg418X), g.3073C>T (p.Gln468X); two flame shift mutations: g.1102_1105delCAGT (p.Gln130SerfsX124), g.2597delC (pThr382SerfsX29); one splicing site mutation: IVS8-1G (p.Val279GlyfsX4); and mutation in start codon: g.2T>C (p.Met1?). Next, they extracted RNA from the lymphoblasts of cases with mutations g.90_91insCTGC (p.Cys31LeufsX34) and c.2923C>T (p.Arg418X), performed quantitative RT-PCR analysis and found that the expression of *GRN* mRNA was reduced by approximately 50%. They performed sequencing of *GRN* mRNAs and found that most of them encoded wild type *GRN*, whereas the mutated type of *GRN* was rarely detected. These results suggested that the mutated mRNA was degraded by NMD. NMD degrades mRNA with a premature termination codon (PTC) which arise from a splicing error or mutation, and thereby prevent production of an abnormal protein (Maquat 2004).

When the lymphoblasts from patients were treated with the NMD inhibitor, cycloheximide, the mutated mRNA was increased. Immunoblotting analysis revealed that the amount of wild type PGRN protein had decreased compared with the controls and mutated PGRN protein was barely detected. They also detected a significant reduction in the amount of mutated mRNA in the brains of patients with the g.2T>C mutation. They suggested that translation of the protein did not occur because the Kozak sequence was disrupted by the g.2T>C mutation.

Cruts and colleagues also identified five novel *GRN* mutations, IVS0+5G>C (now termed IVS1+5G>C), g.3G>A (p.Met1?), g.1094_1095delCT (p.Pro127ArgfsX2), g.1872G>A (p.Ala237TrpfsX4), and g.1087C>T (p.Gln125X).

IVS1+5G>C indicates a point mutation in the intron 1 splice donor site causing intron 1 retention, resulting in nuclear mRNA degradation (Cruts et al. 2006). Sequence analysis of *GRN* in 103 Belgian FTD patients identified this mutation in the eight probands belonging to different branches of the Belgian founder family. An in silico analysis of the IVS1+5G>C mutation predicted an intense decline in the binding efficiency of the U1 snRNP complex.

Next, they analyzed full length *GRN* cDNA from the brains and lymphoblasts of two probands, abnormal transcripts. According to the polymorphism (rs5848) in the 3′ untranslated region of the *GRN* gene, probands were judged C/T heterogeneous (the T-allele is the disease haplotype). However, on sequence analysis of cDNA from their lymphoblasts or brain tissue, only the C allele was observed. These results suggested a complete disappearance of mutated *GRN* mRNA. Immunoblot analysis using an extract from the lymphoblasts of a proband showed PGRN protein reduction. They confirmed loss of mRNA and wild type PGRN protein reduction in the cases of the g.1087C>T (p.Gln125X) mutation. Subsequently, Gass and colleagues performed systematic screening for the *GRN* gene in 378 FTLD and 48 ALS cases at the Mayo Clinic and identified 23 *GRN* mutations in 39 FTLD cases.

Twenty of these twenty-three mutations (4 nonsense mutations, 12 frame shift mutations and 4 splicing donor site mutations) predicted production of PTC and mutated mRNA degradation by NMD. They also identified novel mutations in the splicing donor site of exon 1 (IVS1+1G>A) as well as a missense mutation (g.26C>A (p.Ala9Asp)). In this study, no mutation was identified in ALS cases. RT-PCR analysis of a brain with an IVS1+1G>A mutation revealed two bands corresponding to mutated *GRN* mRNA and wild type *GRN* mRNA, respectively. These results suggested that the IVS1+1G>A mutation did not cause degradation of mutated mRNA by NMD. Initiation of NMD first required a translation process, so that it has been speculated that any IVS1+G>A mutation would escape NMD because no translation would start without the Kozak sequence. The g.26C>A (p.Ala9Asp) mutation was identified as singular missense mutation in this study, the 9th alanine in exon 1 of *GRN* being replaced by aspartic acid. The 9th alanine corresponds to the hydrophobic core of the signal peptide. Mutated mRNA was reduced in the g.26C>A (p.Ala9Asp) brain by an unknown mechanism. If a mutated allele was translated in this case, it would produce a mutated PGRN protein lacking binding capability to the signal recognition motif and could not be transported to the endoplasmic reticulum. Since 2006, many novel *GRN* mutations have been found and are listed in Tables 1, 2 and 3.

PGRN Protein Is Not a Component of Ubiquitin-Positive Inclusion Bodies

Immunohistochemical staining using antibodies for all regions of PGRN protein showed that some of the neurons and activated microglia were positive. Ubiquitin-positive neuronal cytoplasmic inclusions (NCI) and neuronal intranuclear

inclusions (NII) were negative with PGRN antibodies (Baker et al. 2006; Cruts et al. 2006). These results indicated that PGRN accumulation did not occur during development of the FTD pathology caused by the *GRN* mutation. PGRN-positive neuron and activated microglia were also observed in the brains of normal elderly individuals and Alzheimer's disease (AD) cases.

Clinico-Pathological Characterization of *GRN* Mutation Carriers

Incidence Rate

In the Belgian study, Cruts and colleagues found *GRN* mutations in 10.7% (11 out of 103) of the FTD cases overall and in 25.6% (11 out of 43) of familial FTD cases (Cruts et al. 2006). MAPT mutation frequencies were 2.9% (3 out of 103) in the non-familial FTD and 7% (3 out of 43) in the familial FTD cases. These results indicated that *GRN* mutations are approximately a 3.5 times more frequent cause of FTD in Belgian patients. *GRN* mutation data of Gass and colleagues showed mutations in 10.5% (39 out of 378) of FTD and 25.6% (32 out of 144) of familial FTD cases. However, they pointed out that there was some bias in their cases because the Mayo Clinic treated many familial FTLD patients or FTLD patients with a definitive pathological diagnosis.

To exclude this kind of clinical bias, 167 non-selective FTLD cases were collected between 1990 and 2006 in five different Alzheimer's disease research centers and analyzed. The frequency of the *GRN* mutation was 48%. It was noted that the frequencies of the *GRN* and *MAPT* mutations were almost the same; the frequency of the *MAPT* mutation was 44% in the same series of brains. Further investigation of this similarity will be needed. Of 649 dementia cases collected in Minnesota between 1987 and 2006 as part of a dementia research project, 15 were diagnosed with FTLD. Three patients were identified with the *GRN* mutation. The frequency of the *GRN* mutation in the dementia patients overall was calculated to be 0.5%.

Pickering-Brown and colleagues reported that the frequency of the *GRN* mutation was 7.3% (14 out of 192 FTLD patients) (Pickering-Brown et al. 2008) whereas Le Ber and colleagues reported the frequency to be 6.4% (32 out of 502 FTD patients) (Le Ber et al. 2008). The frequency of *GRN* mutations in probands was 5.7% (20 of 352) in fvFTD, 4.4% (3 of 68) in primary progressive aphasia (PPA) and 3.3% (1 of 30) in corticobasal syndrome (CBS). The authors also mentioned that no mutations were found in the 52 probands with FTD-MND. Yu et al. found the frequency of the *GRN* mutation to be 6.9% (30 of 434) (Yu et al. 2010).

Age of Onset

The age of onset of FTLD in Belgian patients with the IVS1+5G>C mutation was 45–70 years (average 63.4 ± 6.8) (Cruts et al. 2006). This mutation was identified in a few asymptomatic individuals; one who had died at 41 years of age, two who had died within the normal age of onset at ages (44 and 54 years) and the one who died at 81. Gass et al. found that the age of onset was 48–83 years (average 59.0 ± 7.0) among *GRN* mutation carriers over all (Gass et al. 2006). Other studies demonstrated that the average age of FTLD onset was 59.0 ± 5 (Pickering-Brown et al. 2008); 59.4 ± 9.4 in FTD, 62.0 ± 7.9 in FTD-MND, 63.8 ± 8.5 in PPA and 61.8 ± 9.7 in CBS (Le Ber et al. 2008). In another study, the average age of onset was 57.7 years, which was calculated from the onset age of 31 *GRN* mutation-positive patients from 28 different families (Yu et al. 2010). Leverenz et al. investigated two families with the *GRN* c.709-2A>G mutation (now termed g.1871A>G (p.Ala237TrpfsX6)) (Leverenz et al. 2007). In family 1, the mean age of onset was 55.6 ± 8.9 years (range = 35–69), the mean age at death was 65.5 ± 6.8 years (range = 56–78) and the mean duration was 9.8 ± 5.5 years (range = 4–22). In family 2, the mean age of onset was 61.0 ± 6.6 years (range = 50–67), the mean age at death was 68.6 ± 6.0 years (range = 57–73) and the mean duration was 6.8 ± 0.4 years (range = 6–7) (Leverenz et al. 2007).

Clinicopathological Images of FTLD

Patients with the *GRN* IVS1+5G>C mutation show non-fluent aphasia (Cruts et al. 2006). Gass et al. indicated that FTLD patients with the *GRN* mutation often exhibited dysphasia and this was rarely accompanied by motor neuron dysfunction (Gass et al. 2006). Pathological analysis of *GRN* IVS1+5G>C patients revealed the presence of neuronal cytoplasmic inclusions (NCIs). Neuronal intranuclear inclusions (NIIs) were also observed in all cases. These observations corresponded with previous reports in which NIIs were commonly detected in familial FTLD patients without motor neuron dysfunction (Mackenzie and Feldman 2003; Woulfe et al. 2001). These results suggested that NIIs would be a pathological marker of PGRN mutation cases. However, NIIs were also found in sporadic FTLD cases or FTLD patients with motor neuron dysfunction, indicating that more investigation will be needed (Mackenzie et al. 2006a). Investigating the clinical response to the *GRN* mutation, Gass et al. found that the most common diagnosis was FTD followed by PA. Other diagnoses were CBD, AD with convulsions and motor dysfunction (PD, parkinsonism and FTD-MND) (Gass et al. 2006). Snowden and colleagues reported that in a single pedigree of the g.3073C>T (p.Gln468X) mutation, patients showed

symptoms of FTD and PA (Snowden et al. 2006). Masellis et al. reported that a patient with the *GRN* IVS7+1G>A (p.Val200GlyfsX18) mutation exhibited CBD-like symptoms (Masellis et al. 2006). Pickering-Brown et al. reported that in patients with the *GRN* mutation, 57% were diagnosed as FTD, 36% as PNFA and 7% as apraxia and parkinsonism (Pickering-Brown et al. 2008). Le Ber et al. reported that 63% of patients with the GRN mutation were diagnosed as fvFTD with other clinical patterns being PPA (16%), CBS (6%) and Lewy body disease (LBD) (6%) (Le Ber et al. 2008). They also found that 9% of patients had other diagnoses including AD and parkinsonism (Le Ber et al. 2008). The most common diagnosis was FTD including PPA and CBS. Other clinical phenotypes such as AD, AD+PD and LBD were observed (Yu et al. 2010).

Immunohistochemical analyses for phosphorylated TDP-43 revealed a considerable number of neuronal cytoplasmic inclusions and dystrophic neurites in *GRN* mutation cases (Fig. 1). In FTLD-TDP, TDP-43 pathology falls within four histological subtypes (types A-D) based on the predominant type of TDP-43-positive structures exhibited (Mackenzie et al. 2011). Type A is characterized by numerous short dystrophic neurites (DNs) and crescentic or oval shaped neuronal cytoplasmic inclusions (NCIs). Cases of FTLD-TDP with a *GRN* mutation invariably display type A pathology (Cairns et al. 2007; Josephs et al. 2007; Mackenzie et al. 2006b).

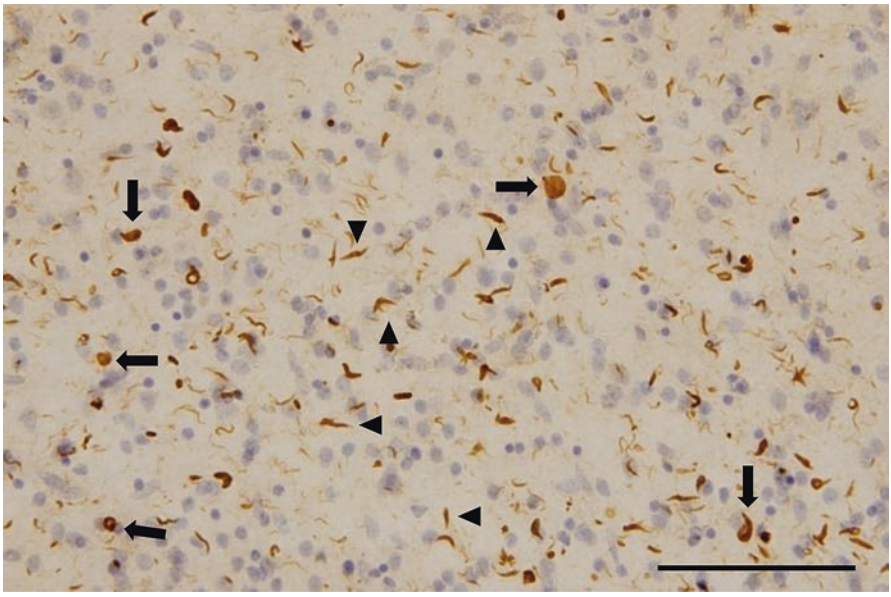

Fig. 1 Immunohistochemical staining of the temporal lobe of a *GRN* mutation case with antibody to phosphorylated TDP-43. Numerous neuronal cytoplasmic inclusions (arrows) and dystrophic neurites (arrowheads) were stained with anti-TDP-43-pS409/410 antibody and the section was counterstained with hematoxylin. Scale bar = 100 μm

PGRN Protein Levels in *GRN* Mutation Carriers

Plasma PGRN protein levels were measured in FTLD patients with the g.1975_1978delCTCA (p.Leu271LeufsX10) mutation or the g.2473 C>T (p.Gln341Arg) mutation and in unaffected individuals with the g.1975_1978del CTCA (p.Leu271LeufsX10) mutation, and in all cases were found to have significantly reduced expression of PGRN (Ghidoni et al. 2008). Plasma PGRN was proposed as a useful biomarker. Sleegers et al. reported that serum PGRN levels were reduced in both affected and unaffected carriers of the PGRN null mutation (IVS1+5G>C) compared with their noncarrier relatives (Sleegers et al. 2009). The authors also measured serum PGRN levels in carriers of the g.1129T>C (p.Cys139Arg) and g.3542C>T (p.Arg564Cys) mutations, and found them to be significantly lower than in controls, but greater than in null mutation carriers. They concluded that the serum PGRN level is a reliable biomarker for diagnosis of FTLD caused by a PGRN null mutation (Sleegers et al. 2009).

Plasma PGRN levels were measured in PGRN loss-of-function mutation carriers, FTLD patients without *GRN* mutations or symptomatic/asymptomatic *GRN* mutation carriers (Finch et al. 2009). Pathogenic *GRN* loss-of-function mutations such as g.26C>A (p.Ala9Asp), g.1098_1101delTAGT (p.Gln130SfsX125), g.2273_2274insTG (p.Trp304LeufsX58fs), g.2450delC (p.Gly333ValfsX28), g.3240C>T (p.Arg493X) and g.3175A>G (p.Ala472_Gln584del) resulted in significantly reduced plasma PGRN levels. Missense mutations (g.2422G>A (p.Ala324Thr)), g.2968C>T (p.Arg433Trp), g.3012C>T (p.His447His) and g.3586G>A (p.Pro578Pro) were associated with plasma PGRN levels equal to those of the controls, but g.55C>T (p.Arg19Trp) and g.1129T>C (p.Cys139Arg) cases showed plasma PGRN levels below the level of detection in controls. These results suggested that g.55C>T (p.Arg19Trp) and g.1129T>C (p.Cys139Arg) mutations might induce a partial loss of PGRN function (Finch et al. 2009). Plasma PGRN levels were also lower than those in carriers of the PGRN g.1A>G (p.Met1), g.1129T>C (p.Cys139Arg), p.Ala89ValfsX41 and p.Ala303AlafsX57 mutations (Gomez-Tortosa et al. 2013).

Mean plasma PGRN levels within the FTLD group were significantly lower in patients with *GRN* mutations than in those with *C9ORF72* expansions, or those without mutation (Gibbons et al. 2015). Meeter and colleagues recently reported that PGRN levels in the plasma and CSF of patients with a loss-of-function *GRN* mutation (g.366delC (p.Ser82ValfsX174), g.1087C>T (p.Gln125X), g.1102_1105delCAGT (p.Gln130SerfsX125) and g.2902_2903delGT (p.Val411SerfsX2)) and presymptomatic loss-of-function *GRN* mutation carriers were lower than those of healthy controls (Meeter et al. 2016).

It has been reported that the homozygous carriers of the T-allele of rs5848 have an elevated risk of developing FTD. TT genotype carriers had lower serum PGRN levels than CT or CC carriers (Hsiung et al. 2011). The rs5848 T-allele is known to be a miRNA-659 binding site and rs5848 may enhance translational inhibition of *GRN* and alter the risk of FTD and other dementias (Hsiung et al. 2011).

The Effect of *GRN* Mutation and Its Influence on PGRN

The effects of *GRN* mutation and its influence on PGRN function are as follows.

1. Mutations that introduce a premature termination codon (PTC) induce nonsense-mediated mRNA decay machinery.
2. Mutations in the intron 1 splice-donor site such as IVS1+3A>T and IVS1+5G>C may generate intron 1 read-through mRNA. Such aberrant mRNAs may not be capable of normal transport through the nuclear pore complex, so that they may remain in the nuclear area where they are then liable to be degraded by the nuclear mRNA degradation system.
3. Complete gene deletion such as found in del*GRN* (Gijselinck et al. 2008) or g.-95_3490del in French patients (Rovelet-Lecrux et al. 2008) may lead to no PGRN at all.
4. Missense mutations in the signal peptide may induce mislocalization of PGRN and insufficient translocation to the endoplasmic reticulum (ER).
5. Missense mutations in other areas may also cause problems. If the mutations exist in the consensus sequence of PGRN, they may be pathological because aberrant protein folding may occur in the ER and reduce PGRN secretion to the extracellular lumen. However, the pathological nature of almost all of them is unknown. The other missense mutations are considered to be benign.

GRN Mutation: Multiple Proteinopathy?

GRN mutations were initially found in tau-negative patients (Baker et al. 2006) (Cruts et al. 2006), but recent findings indicate that these mutations are associated with other neurodegenerative disorders with tau pathology, including AD and CBD. Leverenz et al. found that families with the *GRN* g.1871A>G (p.Ala237TrpfsX6) mutation had variable clinical presentations such as PD, AD, HD, depression and schizophrenia (Leverenz et al. 2007). Immunohistochemical analyses revealed that six of seven cases had evidence of distinctive tau pathology and two of the seven cases also had α-synuclein pathology (Leverenz et al. 2007).

A reduction in progranulin in tau transgenic mice was associated with an increasing tau accumulation (Hosokawa et al. 2015). A reduction in progranulin in APP transgenic mice was associated with a decrease in Aβ accumulation (Takahashi et al. 2017; Hosokawa et al. 2018).

Human *GRN* mutation cases were investigated histochemically and biochemically by Hosokawa and colleagues. Results showed a neuronal and glial tau accumulation in 12 of 13 *GRN* mutation cases (Hosokawa et al. 2017). Tau staining revealed neuronal pretangle forms and glial tau in both astrocytes and oligodendrocytes. Furthermore, phosphorylated α-synuclein-positive structures were also found in oligodendrocytes as well as in the neuropil. Immunoblot analysis of fresh frozen brain tissues revealed that tau and α-synuclein were present in the sarkosyl-insoluble

fraction and were composed of three- and four-repeat tau isoforms, resembling those found in AD. These data suggested that PGRN reduction might be the cause of multiple proteinopathies due to the accelerating accumulation of abnormal proteins. These might include TDP-43 proteinopathy, tauopathy and α-synucleinopathy (Hosokawa et al. 2017).

Very recently, Sieben and their colleagues reported that a family with a *GRN* loss-of-function mutation (IVS1+5G>C) had tau and α-synuclein pathology (Sieben et al. 2018). Of nine members of this family, all were tau-positive and one case had extensive Lewy body pathology. No Aβ pathology or mild accumulation was observed (Sieben et al. 2018).

Recent findings have suggested that different clinical phenotypes may occur in carries of the same *GRN* mutation and additional tau or α-synuclein accumulation may be observed. It has been also reported that PGRN deficiency causes lysosomal dysfunction (Tanaka et al. 2017). Lysosomal dysfunction may reduce protein degradation in brain cells, allowing aggregation-prone neurodegenerative disease-related proteins to deposit more easily (Hosokawa et al. 2017).

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PGRN and Neurodegenerative Diseases Other Than FTLD

Masato Hosokawa

Abstract Progranulin (PGRN) is a multi-functional protein which acts to promote neuronal cell growth and to reduce inflammation in the brain. *Granulin* (*GRN*) mutations were first identified in frontotemporal dementia patients with ubiquitin-positive, tau-negative brain inclusions. However, *GRN* mutations and *GRN* polymorphisms (rs5848) have been found in neurodegenerative diseases other than FTD, such as Alzheimer's disease (AD), Parkinson's disease (PD), corticobasal syndrome (CBS), and amyotrophic lateral sclerosis (ALS). Recent studies showed that phosphorylated TDP-43, tau and α-synuclein accumulation was present in the brains of patients with a *GRN* mutation. These results suggested that *GRN* mutations might cause multiple proteinopathies of which the mechanisms remain unknown.

Keywords Alzheimer's disease (AD) · Corticobasal syndrome (CBD) · Frontotemporal dementia (FTD) · Haploinsufficiency · Parkinson's disease (PD) · rs5848 polymorphism

Introduction

Progranulin (PGRN), formerly known as acrogranin, glycoprotein 88 kD (GP88), granulin-epithelin precursor or PC cell-derived growth factor, is a growth factor which is encoded on chromosome 17q21. PGRN is a 593-amino acid, cysteine-rich protein with a 17 amino acid signal peptide and highly conserved 7.5 tandem granulin repeats of a 12 cysteinyl motif, and is thought to control multiple functions, including wound healing (He et al. 2003; Zhu et al. 2002), inflammation (Yin et al. 2010) and neuronal cell growth (Daniel et al. 2003; Van Damme et al. 2008). It has also been strongly related to tumorigenesis (Ong and Bateman 2003). Moreover, it acts as a chemoattractant for microglia (Pickford et al. 2011). Two research groups

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identified granulin *(GRN)*-null mutations in familial frontotemporal dementia (FTD) linked to chromosome 17q21 with tau-negative, ubiquitin-positive inclusions (Baker et al. 2006; Cruts et al. 2006). There have also been reports that many mutations result in premature termination codons (PTCs), including mutations that involve nucleotide substitution or frame shift by insertion/deletion. *GRN* haploinsufficiency was proposed as being responsible for the loss of functional PGRN protein. A mutation in the signal peptide may induce mislocalization of PGRN in a protein secretion pathway or a PGRN loss of function by a disturbance in its transportation (Gass et al. 2006; Mukherjee et al. 2006). Thus, these types of mutations are strongly involved in FTD pathogenesis. Recently, the relationship between *GRN* mutations/*GRN* polymorphisms and neurodegenerative diseases other than FTD, such as Alzheimer's disease (AD), Parkinson's disease (PD), corticobasal syndrome (CBS), and amyotrophic lateral sclerosis (ALS) have been reported. Additionally, PGRN reduction was observed in some neurological diseases. This section mainly reviews *GRN* mutations and these diseases.

GRN Mutations and Neurodegenerative Diseases

GRN *Mutations and Alzheimer's Disease*

Loss-of-function *GRN* mutations have been confirmed in Alzheimer's disease (AD) patients (Brouwers et al. 2007; Carecchio et al. 2009; Cortini et al. 2008; Finch et al. 2009; Kelley et al. 2009; Le Ber et al. 2008; Leverenz et al. 2007; Piaceri et al. 2018; Rademakers et al. 2007; Sleegers et al. 2008). Of these, g.3366C>T (p.Arg535X) and Null (IVS1+5G>C) mutations were originally identified in FTLD-*GRN* but were also found in AD (Brouwers et al. 2007). Two other mutations found in AD, g.1129T>C (p.Cys139Arg) and g.3023 C>T (p.Pro451Leu), were shown to be pathogenic based on results of an evolutionary conservation and in silico protein modeling study (Brouwers et al. 2008). The mutations g.103G>A (p.Gly35Arg) (Cortini et al. 2008), and another with a single base pair deletion, g. 277delA (p.Thr52HisfsX2), were also discovered in AD, and g.277delA was shown to induce a frame shift creating a PTC (Kelley et al. 2010). Very recently, Redaelli et al. reported an intriguing case study of fraternal twins carrying the *GRN* g.1129T>C (p.Cys139Arg) mutation. They found neurofibrillary pathology (Braak stage V) and massive Aβ deposition without the hallmark lesions of a TDP-43 accumulation in AD patients with this mutation (Redaelli et al. 2018). Clinical and neuroimaging features ensured the diagnosis of probable AD in one sister. They performed immunohistochemistry on the other sister with same mutation and found phosphorylated-tau positive neurofibrillary changes and abundant Aβ deposition. Using phosphorylation independent anti-TDP-43 antibody, neuronal cells of both cases showed physiological nuclear staining of TDP-43 in neuronal cells but no aberrant

or cytoplasmic inclusions. Additionally, staining with anti-phosphorylated-TDP-43 antibody did not reveal any pathologic immunoreactive inclusions. They hypothesized that *GRN* missense mutations may elicit or act as a strong risk factor for neurodegenerative diseases other than FTLD-TDP, particularly for AD. The *GRN* g.1129T>C (p.Cys139Arg) mutation is presumably pathological because of its ability to impair elastase cleavage (Wang et al. 2010), and reduce plasma PGRN levels (Piaceri et al. 2014). This mutation was absent in more than 1000 control individuals (Antonell et al. 2012; Piaceri et al. 2014). Piaceri and colleagues also identified two novel *GRN* mutations (p.Cys149LeufsX10 and pTrp304Cys) in Italian families, and the affected patients had AD or FTLD.

GRN *Mutations and Parkinson's Disease*

α-synuclein deposition is observed in the substantia nigra of patients with PD, but the possible relationship between *GRN* mutation and α-synuclein accumulation is unknown. Brouwers and colleagues identified one individual with PD who carried the *GRN*-null mutation (IVS1+5G>C), a mutation which was also seen in AD patients (Brouwers et al. 2007). This patient showed such symptoms as bradykinesia, cogwheel rigidity, discrete resting tremor, hypomimia, postural instability and a shuffling gait, but responded well to levodopa. Neuropathological analysis revealed that the patient's brain showed diffuse Lewy bodies and pathological FTLD-TDP. Leverenz et al. investigated two families with *GRN* g.1871A>G (p.A237WfsX4) and found that this mutation is associated with a variety of diseases such as AD, FTD and PD. Interestingly, most cases showed tau pathology and two cases had α-synuclein pathology. Rovelet-Lecrux and colleagues also identified FTLD in a patient with typical PGRN neuropathology in which there was a nearly complete deletion of the PGRN gene (g.-95_3490del). This deletion was also seen present in a sister presenting with PD (Rovelet-Lecrux et al. 2008). These results suggested that PGRN haploinsufficiency might trigger different patterns of neurodegeneration which could translate into FTLD or PD. One family with the g.2273_2274insTG (p.Trp304LeufsX58) mutation showed PD symptoms (Kelley et al. 2009). Autopsy findings revealed that this family had FTLD-U, neuronal intranuclear inclusions (NII) and coexistent diffuse Lewy body pathology. Carecchio et al. reported a case of PD with a novel mutation in *GRN* (p.Gly387fsX25) (Carecchio et al. 2014). The plasma PGRN concentration in this patient was measured by ELISA and was found to be markedly decreased (13 pg/ml) compared with normal values (> 70 pg/ml) (Carecchio et al. 2014). FTLD patients with parkinsonism are generally poorly or not responsive at all to levodopa (Boeve et al. 2006; Ghidoni et al. 2008), except for patients with the *GRN* IVS1+5G>C mutation (Brouwers et al. 2007). Very recently, Chang et al. reported that a novel *GRN* g.3223C>T (pThr487Ile) mutation in Taiwanese patients with atypical parkinsonian disorders (Chang et al. 2018).

GRN *Mutations and Corticobasal Syndrome*

Corticobasal syndrome (CBS) is a tauopathy characterized by progressive asymmetrical parkinsonism and cognitive impairment and is generally unresponsive to dopaminergic treatment, and *GRN* mutations were also found this disease (Benussi et al. 2008, 2009; Coppola et al. 2012; Dopper et al. 2011; Gass et al. 2006; Masellis et al. 2006; Spina et al. 2007). The first indication of a relationship between *GRN* mutations and familial CBS with underlying FTD-U inclusion pathology was reported by Masellis et al. (2006) whose study was published just before TDP-43 was found in FTLD patients. In this study, the splice donor site mutation in the *GRN* IVS7+1G>A (p.Val200GlyfsX18) was found in a Canadian family of Chinese origin. This mutation segregated with the disease in two affected family members and was not found in the 200 control individuals. RT-PCR analysis revealed that the *GRN* IVS7+1G>A mutation was not associated with aberrant PGRN transcripts.

Since then, many mutations have been reported in CBS patients with the *GRN* mutation. One of these was a novel splice donor site mutation in *GRN* IVS8-G>C (pVal279GlyfsX5) (Gass et al. 2006). Other mutations were found in the *GRN* coding region, such as g.26C>A (p.Ala9Asp) (Spina et al. 2007), g.2264_2265insGT (p.Ser301CysfsX61) (Guerreiro et al. 2008), g.1977_1980delCACT (p.Thr272SerfsX10) (Benussi et al. 2008, 2009), g.552dup [c.314dup] (p.Cys105TrpfsX13) (Dopper et al. 2011) and g.1283_1289delCTGCTGT (p.Cys157LysfsX97) (Coppola et al. 2012). Spina et al. investigated a patient with the *GRN* g.26C>A (p.Ala9Asp) mutation. The proband presented with spontaneous left arm levitation and asymmetric parkinsonism. This case exhibited a large number of TDP-43 immunoreactive neuronal cytoplasmic inclusions, dystrophic neurites and neuronal intranuclear inclusions in the frontal cortex. Interestingly, a few phosphorylated tau-positive neurons and neurites were observed in the locus coeruleus, transentorhinal cortex and frontal cortex. In this case, no Aβ or α-synuclein deposition was noted (Spina et al. 2007). Guerreiro and colleagues identified the *GRN* g.2264_2265insGT (p.Ser301CysfsX61) mutation in a Portuguese family. The proband and the proband's sisters and brother all showed obvious features of corticobasal degeneration including unilateral parkinsonism, memory disturbance and progressive dementia. The neuropathological examination of the proband revealed numerous TDP-43-positive neurites and neuronal cytoplasmic inclusions were seen in his brain. Immunohistochemical staining of Aβ, tau and α-synuclein were negative (Guerreiro et al. 2008).

Benussi et al. identified the *GRN* g.1975_1978delCTCA (p.Leu271LeufsX10) mutation in two Italian families. The major neurological findings in affected members of one family were language dysfunction, behavioral abnormality and parkinsonism. The main symptoms in the other family were behavioral abnormality and language dysfunction, and some of them showed parkinsonism (Benussi et al. 2008). The *GRN* mRNA expression levels were decreased by 50% in an atypical, symmetrical CBS patient with TDP-43 pathology and the *GRN* g.552dup [c.314dup] (p.Cys105TrpfsX13) mutation (Dopper et al. 2011).

GRN *Mutation and Amyotrophic Lateral Sclerosis (ALS)*

Sleegers and colleagues sequenced the *GRN* gene in a Belgian study of 230 patients with ALS and 436 healthy control individuals. They also sequenced the *GRN* gene of 308 Dutch patients with ALS and 345 controls (Sleegers et al. 2008). They identified four missense *GRN* mutations in exons of patients with ALS: g.567G>A (p.Arg110Gln), g.1085T>C (p.Ile124Thr), g.2422G>A (p.Ala324Thr) and g.2924G>A (p.Arg418Gln), two benign mutations; g.1128G>A (p.Thr138Thr) and g.3012C>T (p.His447His) and one polymorphism; g.104025G>A. Other mutations were identified in control individuals [g.99C>T (p.Asp22Asp), g.1098T>C (p.Asp128Asp), g.1968G>A (p.Thr268Thr), g.2266G>A (p.Ser301Ser)]. These missense mutations were positioned in or at the border of a granulin domain, and three of these [g.567G>A (p.Arg110Gln), g.2422G>A (p.Ala324Thr), g.2924G>A (p.Arg418Gln),] were not highly conserved residues in the wild-type. The *GRN* g.1085T>C (p.Ile124Thr) mutation is located at a moderately conserved position between two granulin domains. SIFT analysis (Kumar et al. 2009), which predicts whether an amino acid substitution affects protein function, indicated that the g.567G>A (p.Arg110Gln), g.2422G>A (p.Ala324Thr), and g.2924G>A (p.Arg418Gln) mutations were unlikely to affect protein function, but that the g.1085T>C (p.Ile124Thr) would. Moreover, Sleegers and colleagues found rare alleles of IVS2+21G>A (rs9897526), IVS3-47_46insGTCA (rs34424835), and IVS4+24G>A (rs850713). These alleles correlated with age at the onset of ALS and the subsequent years of survival. They concluded that PGRN haploinsufficiency does not contribute significantly to ALS pathogenesis but that genetic variability in *GRN* may modify the disease progression. The mutations g.1073C>A (p.Ser120Tyr), g.1360C>T (p.Thr182Met), g.26228A>C (p.Pro392Pro) and g.3317C>T (p.Asp518Asp) were identified in ALS patients (Guerreiro et al. 2010), but their pathogenic role was uncertain. Schymick et al. identified a g.1073C>A (p.Ser120Tyr) mutation in an ALS-FTD patient and a g.1360C>T (p.Thr182Met) mutation in a single case of sporadic ALS with limb onset. The pathogenicity of these variants remains to be elucidated (Schymick et al. 2007).

GRN rs5848 Polymorphism and Neurodegenerative Diseases

GRN *rs5848 Polymorphism and Alzheimer's Disease*

No association of rs5848 (3′UTR+78C>T) with FTLD was observed in two independent studies (Rollinson et al. 2011; Simon-Sanchez et al. 2009). These data suggested that the rs5848 is not a risk factor for FTLD. However, a rs5848 variant was also found in AD (Fenoglio et al. 2009) and was associated with an increased risk of this disease (Lee et al. 2011). The rs5848 variant in the 3′ untranslated region

of *GRN* is known to reduce *GRN* mRNA levels in the brains and peripheral mononuclear cells of AD patients. Thus, this variant would result in a reduced PGRN protein level with an increased risk for AD. However, the rs5848 risk variant (T allele) had no effect on florbetapir-PET amyloid imaging or CSF $A\beta_{42}$ levels in the Alzheimer's disease neuroimaging initiative (ADNI) participants (Takahashi et al. 2017). These data suggested that the T allele of the rs5848 had no detectable influence on Aβ pathology in AD. Interestingly, the rs5848 risk variant is associated with upregulation of total tau levels in CSF but does not have a significant effect on CSF p-tau181 (Takahashi et al. 2017). Generally, the total tau and phosphorylated tau (pT181) levels in CSF are increased in AD patients (Fagan et al. 2009; Hampel et al. 2004; Sunderland et al. 2003).

GRN *rs5848 Polymorphism and Parkinson's Disease*

The rs5848 variant is not considered a risk factor for PD (Jasinska-Myga et al. 2009). However, compared with the CC genotype, the TT genotype represents a 1.58-fold increased risk of PD in a Taiwanese study of 573 PD patients and 490 age-matched control individuals (Chang et al. 2013). Notably, this morbidity was observed in females, in which the TT genotype increased the risk of PD 2.16-fold as compared with the controls. These results suggested that the rs5848 TT genotype and the T allele are risk factors for PD in Taiwanese females (Chang et al. 2013). Mateo and colleagues reported that the concentration of PGRN in PD serum was significantly lower than that of controls. However, there was no correlation noted between rs5848 genotypes and serum PGRN concentrations (Mateo et al. 2013).

GRN *rs5848 Polymorphism and Hippocampal Sclerosis*

Hippocampal sclerosis (HpScl) is a pathological diagnosis that is classically defined by selective neuronal loss and gliosis in hippocampal CA1, as observed in the brains of epileptics. Similar observations have been made in older adults with dementia (Dickson et al. 1994). HpScl was formerly defined as damage caused by hypoxic or ischemic injury to the hippocampus. More recently, it has been correlated to neurodegenerative processes (Amador-Ortiz et al. 2007; Probst et al. 2007; Zarow et al. 2008). Interestingly, the rs5848 T-allele responsible for lowering levels of PGRN might be a risk factor for HpScl in AD (Dickson et al. 2010). Further investigation is needed to determine the relationship between rs5848 polymorphism and HpScl.

PGRN Reduction and Neurological Diseases

PGRN and Gaucher Disease

Gaucher disease (GD) is a lysosomal storage disease that is induced by mutations in the *GBA1* gene encoding of β-glucocerebrosidase (GCase). *GBA1* mutations initiate defective GCase function and the consequent accumulation of its substrate, glucosylceramide, in cells. GD is subdivided into three groups: type 1, non-neuropathic; type 2, acute neuropathic; and type 3, chronic neuropathic. Jian and colleagues investigated the relationship between PGRN and GD, and showed that serum PGRN levels were significantly lower in GD patients with the p.Asn370Ser mutation in *GBA1* compared with healthy controls (Jian et al. 2016b). The genotyping of four SNPs (rs4792937, rs78403836, rs850713 and rs5848) identified in GD patients revealed that the four SNP sites are found with a significantly higher frequency in GD patients. Moreover, old and OVA-challenged *Grn*-KO mice showed GD-like phenotypes. Interestingly, recombinant PGRN administration improved GD-like phenotypes in mice as well as human fibroblasts from GD patients (Jian et al. 2016a). They showed that PGRN bound directly to GCase and recruited heat shock protein 70 (HSP70). PGRN may act as a co-chaperone of HSP70 and play a pivotal role in GCase lysosomal localization (Jian et al. 2016a).

GRN and Bipolar Disorders

The relationship between PGRN and bipolar disorders (BPD) was studied by Galimberti and colleagues. They investigated plasma PGRN levels in 26 German BPD patients, 61 Italian BPD patients and 29 matched controls. The results indicated that plasma PGRN levels were significantly decreased in BPD patients compared to the controls (Galimberti et al. 2012). They also investigated whether *GRN* genetic variability (rs2879096, rs4792938, rs5848) could decrease the risk of developing BPD and schizophrenia. Their replication study (Galimberti et al. 2014) showed similar results. Another independent study was conducted by Kittel-Schneider and colleagues (Kittel-Schneider et al. 2014). They showed that plasma PGRN levels were decreased in BPD patients and that the rs5848 variant was associated with plasma PGRN levels.

Relationship Between Aβ/tau/α-synuclein Deposition and PGRN

Gliebus et al. reported that PGRN forms plaque-like structures in conditions associated with Aβ aggregation in a AD patient with a presenilin-1 mutation (p.Ala246Glu) (Gliebus et al. 2009). These structures most frequently colocalized with Aβ. These findings suggested that a biological association between Aβ and PGRN might exist. In AD mouse models, PGRN levels correlated significantly with the amyloid load,

especially with dense-core plaque pathology (Pereson et al. 2009). They also showed that PGRN is upregulated in microglia, neurons and neurites around dense-core plaques. The strong PGRN immunoreactivity around dense-core plaques may be indicate that PGRN has an important role in AD pathogenesis (Pereson et al. 2009).

Minami et al. reported that a loss of PGRN might increase Aβ deposition in APP^{high} LysM-cre^{+}*Grn*$^{flox/flox}$ mice (Minami et al. 2014). Recently, Takahashi et al. reported that an overall PGRN decline elicited microglial TYROBP network gene expression and increased the AD risk by contributing to neuronal cell damage and tau deposition, rather than by facilitating Aβ deposition (Takahashi et al. 2017). They also showed that a PGRN deficiency *(Grn*$^{-/-}$*)* prevents diffuse Aβ plaque growth in APP/PS1 mice. Moreover, PGRN haploinsufficiency *(Grn*$^{+/-}$*)* also reduced Aβ deposition in APP transgenic mice (Hosokawa et al. 2018) and Aβ deposition was either absent or relatively mild in human FTLD cases with the *GRN* mutation (Hosokawa et al. 2017; Sieben et al. 2018). These results indicated that a PGRN decrease by a *GRN* mutation may not be causative of or a risk factor for Aβ pathology. On the other hand, according to the level of tau accumulation, PGRN haploinsufficiency *(Grn*$^{+/-}$*)* in tau transgenic mice may accelerate tau deposition caused by activation of cyclin dependent kinases (Hosokawa et al. 2015). Moreover, a PGRN deficiency *(Grn*$^{-/-}$*)* exacerbated tau pathology in human P301L tau mice (Takahashi et al. 2017). These results suggest that a reduction in PGRN may lead to abnormal tau deposition. Importantly, some human studies revealed phosphorylated tau accumulation in addition to TDP-43 deposition in FTLD brains with the *GRN* mutation (Fig. 1a) (Hosokawa et al. 2017; Leverenz et al. 2007; Sieben et al. 2018).

To compare biochemical characteristics of deposited tau in *GRN* mutation cases with CBD, PSP and AD, immunoblot analysis of the sarkosyl-insoluble fraction was performed using C-terminal, non-phosphorylated tau antibody (Fig. 2). The triplet bands of 60, 64, and 68 kDa detected in *GRN* mutation cases were similar to those of AD, but dissimilar from those of in CBD and PSP (Fig. 2).

Some brains of FTLD cases associated with a *GRN* mutation were immunopositive for phosphorylated α-synuclein (Fig. 1b). The relationship between PGRN reduction and α-synuclein deposition needs further investigation.

These opposing influences of a *GRN* deficiency on Aβ and tau deposition might be explained as follows. PGRN is elaborated by microglia and PGRN abrogates the hyper-activation of microglia by an autocrine secretion (Tanaka et al. 2013). PGRN deficiency may induce microglial activation and once activated, they may accelerate phagocytic activity and reduce extracellular Aβ (Takahashi et al. 2017). A PGRN deficiency was shown to lead to lysosomal dysfunction (Tanaka et al. 2017) in neurons and may result in abnormal tau or α-synuclein deposition. A schematic illustration of the opposing influence of a *GRN* defect on Aβ and tau is shown in Fig. 3.

The discrepancy among these results might be accounted for by the difference in the mouse strains used, as indicated in Takahashi et al. (2017). APP^{high} LysM-cre^{+}*Grn*$^{flox/flox}$ conditional mice were used in Minami's study and these LysM-cre mice were deprived of endogenous *Lyz2*, which is prominently augmented in *Grn*$^{-/-}$ mice (Lui et al. 2016; Rosen et al. 2011). Microglia from *Grn*$^{-/-}$ mice showed an upregulation of phagocytic activity (Tanaka et al. 2013), however, this activity might be downregulated in APP^{high} LysM-cre^{+}*Grn*$^{flox/flox}$ mice (Minami et al. 2014).

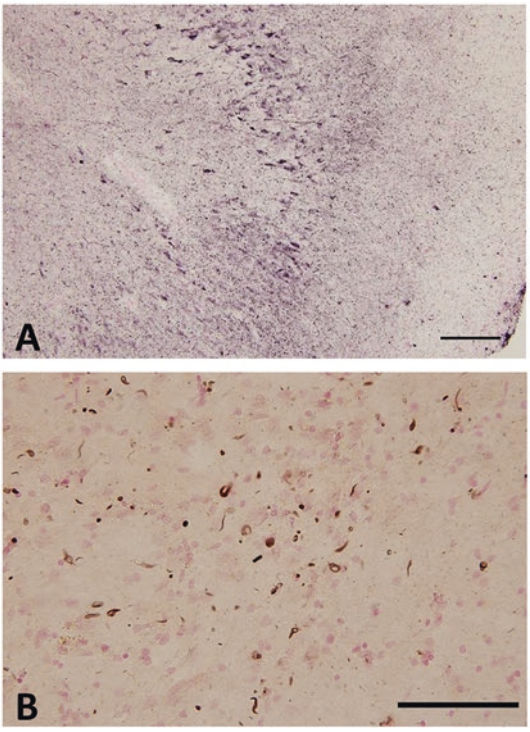

Fig. 1 Immunohistochemical staining of phosphorylated tau in the inferior temporal cortex of a brain with the *GRN* g.3240C>T (p. Arg493X) mutation brain (**a**) and phosphorylated α-synuclein in the inferior temporal cortex of another brain with the same *GRN* g.3240C>T (p.Arg493X) mutation (**b**). The scale bars in (**a**) and (**b**) are 200 μm

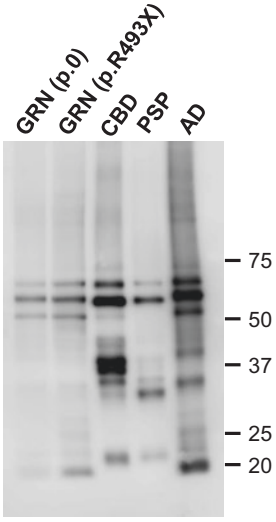

Fig. 2 Comparison of the immunoblotting banding patterns of sarkosyl-insoluble tau of cases with *GRN* mutations and those with other tauopathies. Immunoblotting analysis was performed with the T46 antibody to detect tau in the sarkosyl-insoluble fraction from two cases with a *GRN* g.1A>C (p.0) and a *GRN* g.3240C>T (p.Arg493X) mutation, and one case each of corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and Alzheimer's disease (AD). Molecular weight markers are shown on the right (kDa)

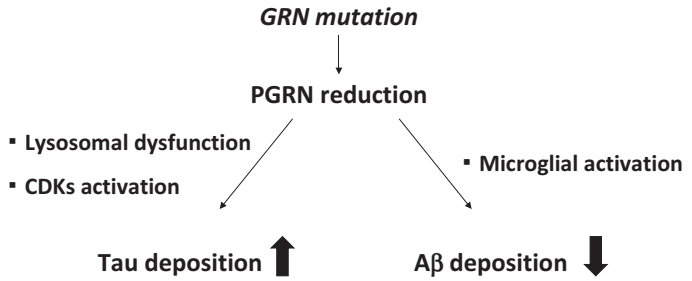

Fig. 3 Illustration of the opposing influence of a *GRN* deficiency on Aβ and tau deposition. PGRN reduction induced by a *GRN* mutation may increase tau accumulation in neurons by CDK activation or lysosomal dysfunction.PGRN diminution may downregulate Aβ deposition by the activation of microglia

To summarize, these reports suggest that *GRN* mutations may be a risk factor for multiple types of dementia-related brain pathologies such as TDP-43 proteinopathy, tauopathy and α-synucleinopathy.

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Progranulin Regulations of Lysosomal Homeostasis and Its Involvement in Neurodegenerative Diseases

Yoshinori Tanaka

Abstract Progranulin (PGRN) haploinsufficiency resulting from the loss-of-function mutations of *GRN* gene causes frontotemporal lober degeneration characteristic of TDP-43-positive inclusion (FTLD-TDP). The patients with homozygous mutations in the *GRN* gene present with adult onset neuronal ceroid lipofuscinosis. While the functional role of PGRN regulating neurodegenerative diseases is still controversial, evidences that PGRN regulates lysosomal function and biogenesis are accumulating. We previously demonstrated that PGRN is localized to lysosomes and the expression increases in lysosomal biogenesis. Furthermore, PGRN suppresses exacerbated lysosomal biogenesis especially in activated microglia after traumatic brain injury and with aging in mice, indicating that PGRN composes of negative feedback loop of lysosomal biogenesis. Interestingly, secreted PGRN is incorporated and transported into lysosomes through sortilin or cation-independent mannose 6-phosphate receptor, and facilitated acidification of lysosomes. These findings indicate that PGRN is a secretory lysosomal protein that regulates lysosomal function and biogenesis through acidification of lysosomes. On the other hand, other groups recently reported that granulin peptides stabilize Cathepsin D and work as a chaperone for beta-glucocerebrosidase. These investigations about PGRN function involved in lysosomes have spotlighted on the pathogenic mechanisms of neurodegenerative diseases especially in FTLD-TDP. The understanding of PGRN trafficking into lysosomes and its regulation of lysosomes could provide a clue of the remedy for currently incurable neurodegenerative diseases.

Keywords Progranulin · Granulin · Lysosome · TDP-43 · FTLD · NCL

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Introduction

Neurodegenerative diseases are characterized by accumulation of protein aggregates including tau, alpha-synuclein, and TAR DNA-binding protein 43 kDa (TDP-43), which define distinct pathology across neurodegenerative diseases (Nonaka et al. 2018). Previous studies indicated that the formation of these aggregates is involved in neuronal cell loss, and these aggregates exhibit characteristic patterns of temporal spreading and topological distribution that correlate with clinical phenotype (Braak and Braak 1991; Braak et al. 2003; Brettschneider et al. 2013). However, it remains elusive when and how these protein aggregation starts. Interestingly, loss-of-function mutations in the *GRN* gene were identified in familial frontotemporal lober degeneration (FTLD) linked to chromosome 17q21 with tau-negative and alpha-synuclein-negative cytoplasmic inclusions (Baker et al. 2006; Cruts et al. 2006), and these inclusions were characterized of enriched TDP-43 (Arai et al. 2006; Neumann et al. 2006). Biochemical and morphological analyses revealed that filamentous, detergent-insoluble, abnormally phosphorylated, partially fragmented and ubiquitinated TDP-43 accumulates in the brain of patients (Arai et al. 2010; Hasegawa et al. 2011). These findings suggested that progranulin (PGRN), the product of *GRN* gene, function is more involved in TDP-43 aggregation process than alpha-synuclein and tau aggregation process.

PGRN is a multifunctional protein involved in a lot of physiological and pathological processes such as inflammation, tumorigenesis and sexual differentiation (Suzuki et al. 1998; He et al. 2002; Kessenbrock et al. 2008). Originally, PGRN was identified as a growth factor containing 7.5 tandem granulin (GRN) motif repeats (Baba et al. 1993; Bateman and Bennett 2009). Since PGRN was identified as a growth factor, cell signalling pathways regulated by PGRN to stimulate cell growth have been studied. While previous studies revealed that PGRN activates typical cell signalling pathways such as ERK, PI3K and Akt pathways (He et al. 2002; Monami et al. 2006; Feng et al. 2010; Gao et al. 2010; Xu et al. 2011), they did not show any cell surface receptors to mediate these effects. On the contrary of the idea that PGRN is a growth factor, the cell surface PGRN receptor firstly detected was sortilin that mediates transportation of target proteins into lysosomes (Hu et al. 2010).

In the brain, the sources of PGRN are supplied from neurons and microglia (Baker et al. 2006; Mackenzie et al. 2006; Mukherjee et al. 2006; Ahmed et al. 2007). Especially, activated microglia predominantly expresses PGRN (Pereson et al. 2009; Naphade et al. 2010; Wang et al. 2010; Byrnes et al. 2011). Microglia is a resident innate immune cell in the brain, and is activated by a stimulus including neuronal damage (Block et al. 2007). Activated microglia is identified as a hallmark of neurodegenerative diseases, because it mediates inflammatory responses in the brain (Zhang et al. 2010). To investigate the role of PGRN in activated microglia, we compared the markers of activated microglia, the level of them increases along with the microglial activation, in wild-type and PGRN-deficient mice adapted to traumatic brain injury (TBI). We found that the level of CD68, a lysosomal protein in the monocyte lineage, increases in PGRN-deficient mice after TBI, suggesting

that PGRN regulates lysosomal homeostasis (Tanaka et al. 2013a). Subsequently, we demonstrated that PGRN localizes to lysosome, and suppresses exacerbated lysosomal biogenesis in activated microglia (Tanaka et al. 2013b). Furthermore, we found that PGRN-deficient mice shows selective vulnerability in the ventral posteromedial nucleus/ventral posterolateral nucleus (VPM/VPL) region of the thalamus, as do model mice of lysosomal diseases (Tanaka et al. 2014; Sargeant 2016). More importantly, PGRN regulates lysosomal function and biogenesis through the acidification of lysosomes (Tanaka et al. 2017). In favour of the PGRN function in lysosomes, patients with homozygous mutations in the *GRN* gene presented adult onset neuronal ceroid lipofuscinosis (NCL) (Smith et al. 2012; Almeida et al. 2016). Moreover, Götzl et al. showed that FTLD patients from PGRN haploinsufficiency also present with typical pathological features of NCL, suggesting that FTLD as well as NCL resulting from PGRN insufficiency is caused by lysosome dysfunction (Gotzl et al. 2014). These findings spotlighted the PGRN function in lysosomes and its protective action on neurodegenerative diseases.

Lysosomes are membrane-bound organelles with acidic compartments containing more than 60 types of hydrolases. The primary function of lysosomes is the degradation of extracellular and intracellular molecules transported by endocytosis, autophagy, and other cellular trafficking pathway (Lubke et al. 2009). On the other hand, there are accumulating evidences that lysosomes are multifunctional organelles involved in secretion, plasma membrane repair, signalling, and energy metabolism (Settembre et al. 2013). Therefore, lysosomal dysfunction from PGRN deficiency could affect a broad range of cellular functions. To clear understanding the relationship between PGRN and lysosomes, and its involvement in neurodegenerative diseases, this chapter focuses on neurodegenerative diseases resulting from PGRN mutations, PGRN trafficking into lysosomes, and PGRN function in lysosomes.

Involvement of Lysosomes in Neurodegenerative Diseases Caused by *GRN* Mutations

FTLD shows progressive atrophy of the frontal and temporal lobes of the brain. FTLD is the second leading cause of early-onset dementia for individuals 65 years and younger, and is the third most common cause of dementia for 65 years and older (Bott et al. 2014). FTLD patients show either significant behavioural deterioration or predominant language decline (Tan et al. 2017). The patients with *GRN* mutations are clinically heterogenous even in family members carrying the same mutation, which makes genotype-phenotype correlations difficult (Chen-Plotkin et al. 2011). However, certain general features of *GRN*-associated FTLD have been recognized. Motor neuron disease appears to be rare in FTLD with *GRN* mutation (Schymick et al. 2007). Then, *GRN*-associated FTLD-TDP is more likely to have parkinsonian features than FTLD-TDP without *GRN* mutations (van Swieten and

Heutink 2008). More interestingly, FTLD patients resulting from PGRN haploinsufficiency show NCL-like features before dementia onset (Ward et al. 2017).

GRN gene was identified as a responsible gene for tau-negative and TDP-43-positive frontotemporal lobar degeneration (FTLD-TDP) linked to chromosome 17q21 (Baker et al. 2006; Cruts et al. 2006). *GRN* mutations have been found to result in 10% of total FTLD cases and 22% of familial FTLD cases (Gass et al. 2006). The majority of these *GRN* mutations is nonsense mutation, and could be a target of the nonsense-mediated mRNA decay pathway (Baker et al. 2006, Cruts et al. 2006). Recently, there are increasing evidences that PGRN regulates lysosomal biogenesis and function (Kao et al. 2017). Taken together, lysosomal dysfunction resulting from loss-of-function of PGRN could be linked to the occurrence of FTLD-TDP. In favour of this, recent studies about *C9orf72* and *VCP*, other causal genes of familial FTLD-TDP, suggest that these genes are associated with the regulation of lysosomal function. Chromosome open reading frame 72 (C9orf72) functions is linked to the degradation of endocytosed material and in the maintenance of lysosomal homeostasis (Corrionero and Horvitz 2018). Valosin containing protein (VCP) is linked to various membrane trafficking processes (Meyer 2005), and several studies connect VCP to lysosomal protein degradation (Ju and Weihl 2010; Bug and Meyer 2012). Moreover, FTLD-linked mutations in genes encoding three autophagy adaptor proteins, p62/SQSTM1, ubiquilin 2, and optineurin indicate that impaired autophagy might cause FTLD (Gotzl et al. 2016). These studies about causal genes of familial FTLD-TDP support the idea that dysfunction of lysosomal system is linked to the occurrence of FTLD-TDP.

While heterozygous mutations of *GRN* cause FTLD, homozygous *GRN* mutations lead to adult onset NCL, a type of lysosomal storage disease. A pair of siblings with homozygous mutations in the *GRN* gene suffering from a newly described type of adult onset NCL was discovered (Smith et al. 2012). In the second case, both parents developed FTLD resulting from the *GRN* mutation, and their daughter who received *GRN* mutations in both allele developed adult onset NCL, indicating that dosage effects of PGRN on the appearance of this disease (Almeida et al. 2016). NCL is caused by mutations at least 14 different genes and characterized by the appearance of a heterogenous origin of the storage material. Commonly, the accumulated ceroid-lipopigments are autofluorescent and are positive for histochemical staining such as periodic acid-Schiff (PAS), Luxol fast blue (LFB), sudan black B. Clinically, they share features such as progressive loss of vision as well as mental and motor deterioration, epileptic seizures, and eventually premature death (Carcel-Trullols et al. 2015). Interestingly, NCL-associated proteins (CLN1 to CLN14) differ in their function and their intracellular localization. 5 NCL types (CLN1, CLN2, CLN5, CLN10, and CLN13) are caused by defects in lysosomal enzymes or proteins, but the localization of other NCL proteins is different from them. For example, CLN6 and CLN8 are localized to Endoplasmic Reticulum (ER), and involved in ER stress response (Marotta et al. 2017). Therefore, the understanding of PGRN function improves to understand how disturbed different NCL proteins lead to similar clinical symptom and neurodegeneration.

PGRN Trafficking into Lysosomes

The findings to suggest the function of PGRN in lysosomes was the discovery that PGRN expression increases along with the activation of microglia, and it often localizes to lysosomes (Naphade et al. 2010; Tanaka et al. 2013a). More importantly, increased lysosomal biogenesis happened to activated microglia in PGRN-deficient mice compared to wild-type mice (Tanaka et al. 2013a, b). These studies shed light on the importance of the mechanism mediating transport of PGRN into lysosome by sortilin, an endocytic receptor to mediate lysosomal trafficking (Fig. 1). It binds to PGRN with a high affinity, and mediated transport of PGRN into lysosome through the endocytic pathway (Hu et al. 2010). While the transport mediated by sortilin is likely to occur at the Golgi apparatus after the synthesis of PGRN, the

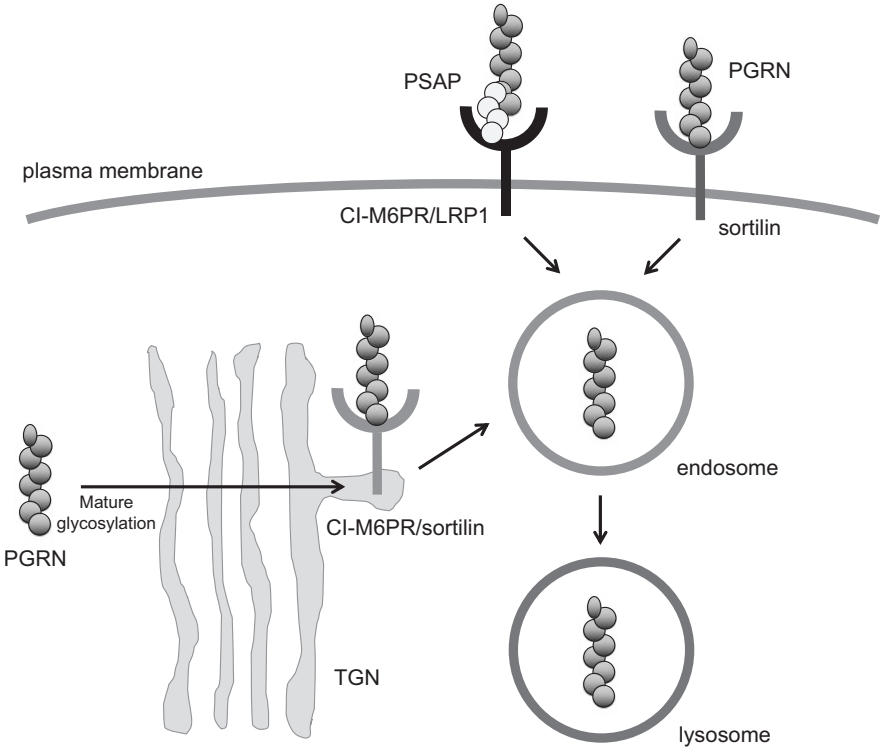

Fig. 1 Lysosomal trafficking of PGRN
PGRN is delivered to lysosomes from extracellular space or TGN. Extracellular PGRN indirectly binds to CI-M6PR/LRP1 through the binding of PSAP or directly binds to sortilin on the plasma membrane. PGRN bound to these receptors is transported to lysosomes through the endocytic pathway. Synthesized PGRN in ER is modified in the ER and Golgi apparatus to display M6P residues and transported to endosomes from TGN by CI-M6PR or sortilin. PGRN in endosomes is transported to lysosomes through the endocytic pathway

level of PGRN in SH-SY5Y cells decreased by the down-regulation of sortilin, suggesting that sortilin is rather important to incorporate extracellular PGRN than transport PGRN produced in the biosynthesis process (Tanaka et al. 2017). Interestingly, while activated microglia dominantly express PGRN in the brain, the sortilin expression is not detected in activated microglia by the immunohistochemistry (Tanaka et al. 2017), suggesting that neurons dominantly incorporate extracellular PGRN from activated microglia. These findings suggest that sortilin is an important receptor for neurons to incorporate extracellular PGRN.

PGRN is a glycosylated protein that has a functional role in lysosomes. Therefore, PGRN might be transported into lysosomes through the common pathway with lysosomal hydrolases (Fig. 1). Hydrolases working in lysosomes are synthesized in the endoplasmic reticulum (ER) and transported to lysosomes from the trans Golgi network (TGN). They get modified in the ER and Golgi apparatus to display mannose-6-phosphate (M6P) residues that are necessary to their specific endolysosomal targeting by M6P receptors (M6PR) (Braulke and Bonifacino 2009). Actually, PGRN transiently expressed in COS7 cells is N-linked glycosylated at least four sites out of five potential PGRN N-glycosylation consensus site (Songsrirote et al. 2010). Interestingly, most of R493X PGRN lack of the sortilin binding site is transported into lysosome as well as wild-type PGRN, suggesting that sortilin-independent mechanism is sufficient to transport PGRN into lysosome (Nguyen et al. 2018). These findings suggest that M6PR directly transport a part of PGRN from the Golgi apparatus to lysosome. To support this idea, the down-regulation of cation-independent M6P receptor (CI-M6PR) increased PGRN levels and suppressed PGRN function in lysosomes, suggesting that CI-M6PR mainly mediate the transport of PGRN produced in the biosynthesis process (Tanaka et al. 2017). Further studies are necessary to investigate the relationship between N-glycosylation and lysosomal trafficking.

On the other hand, another mechanism through indirect binding between PGRN and CI-M6PR or low-density lipoprotein receptor-related protein 1 (LRP1) is shown (Fig. 1). These receptors transport PGRN into lysosomes through the binding of prosaposin (PSAP). Previous studies showed GRN D/E peptide binds to a linker region of saposin B and C (Zhou et al. 2015, 2017c). PSAP is a proprotein composed of four homologous cysteine-rich saposin A, B, C, and D as same as PGRN (Kishimoto et al. 1992). The function of these peptides in lysosomes is to promote sphingolipid hydrolysis (Meyer et al. 2014), and a homozygous loss-of-function mutation in PSAP gene causes a lysosomal storage disease known as sphingolipidoses (Hulkova et al. 2001; Meyer et al. 2014). Interestingly, a previous study showed that PGRN mediates the transport of PSAP into lysosomes through the sortilin (Zhou et al. 2017d). These findings suggest that lysosomal dysfunction from PGRN insufficiency is partly mediated by PSAP or other binding partners, and it's important to understand how the particular pathway involved in PGRN trafficking regulates lysosomal function.

PGRN in Negative Feedback Loop of Lysosomal Biogenesis

FTLD resulting from PGRN haploinsufficiency is characterized by accumulation of TDP-43 aggregates (Arai et al. 2006; Neumann et al. 2006). The accumulation of protein aggregates in neurodegenerative diseases is linked to dysfunction of the proteostasis, which leads to suppress the clearance of protein aggregates and facilitate protein aggregation (Bingol 2018). The histological analysis using aged PGRN-deficient mice suggested that the alteration in lysosomal homeostasis with aging (Ahmed et al. 2010; Wils et al. 2012). Furthermore, complete loss of PGRN led to adult onset NCL (Smith et al. 2012; Almeida et al. 2016), suggesting the association of PGRN with a lysosomal function. We demonstrated that PGRN expression increases after TBI in wild-type mice, and the major source of PGRN is CD68-positive activated microglia. More importantly, PGRN deficiency increased the expression of CD68 in activated microglia (Tanaka et al. 2013a). CD68 is a member of the lysosome-associated membrane protein (LAMP) family that is restrictedly expressed in cells of the monocyte/macrophage lineage, and predominantly localized to late endosomal and lysosomal compartment (Song et al. 2011). Additionally, CD68 gene expression was controlled by transcription factor EB (TFEB), a master regulator of lysosomal biogenesis (Sardiello et al. 2009). Therefore, these findings suggested that PGRN composes of lysosomal biogenesis pathway and is implicated in the regulation of lysosomal biogenesis. Actually, the following study of us showed that increased PGRN in activated microglia is often colocalized with Lamp1, a lysosomal marker. Then, the Lamp1 level increased in activated microglia of PGRN-deficient mice as well as lysosomal gene expression in the affected region. Mammalian target of rapamycin complex 1 (mTORC1) have a dominant role in negatively controlling the translocation of TFEB from the cytoplasm to the nucleus (Settembre et al. 2012). Interestingly, the activity of mTORC1 decreased, and the number of activated microglia with TFEB localized in the nucleus increased in PGRN-deficient mice (Tanaka et al. 2013b). Excessive lysosomal biogenesis also appeared in the aged PGRN-deficient mice (Tanaka et al. 2014). Importantly, aged PGRN-deficient mice exhibit selective vulnerability in the ventral posteromedial nucleus/ventral posterolateral nucleus (VPM/VPL) region of the thalamus, as do model mice of lysosomal diseases (Tanaka et al. 2014; Sargeant 2016). Moreover, FTLD patients resulting from PGRN haploinsufficiency also presented with typical pathological features of NCL (Gotzl et al. 2014). These findings demonstrate that PGRN negatively regulates lysosomal biogenesis *in vivo* (Fig. 2).

Focusing on the regulation of PGRN gene expression, *Grn* gene has two possible CLEAR sequences in the promoter region (Tanaka et al. 2013b), where TFEB binds and thereby increases lysosomal gene expression (Sardiello et al. 2009), suggesting that PGRN works as a key player of the negative feedback loop of lysosomal biogenesis mediated by mTORC1 and TFEB. In favour of this, PGRN gene

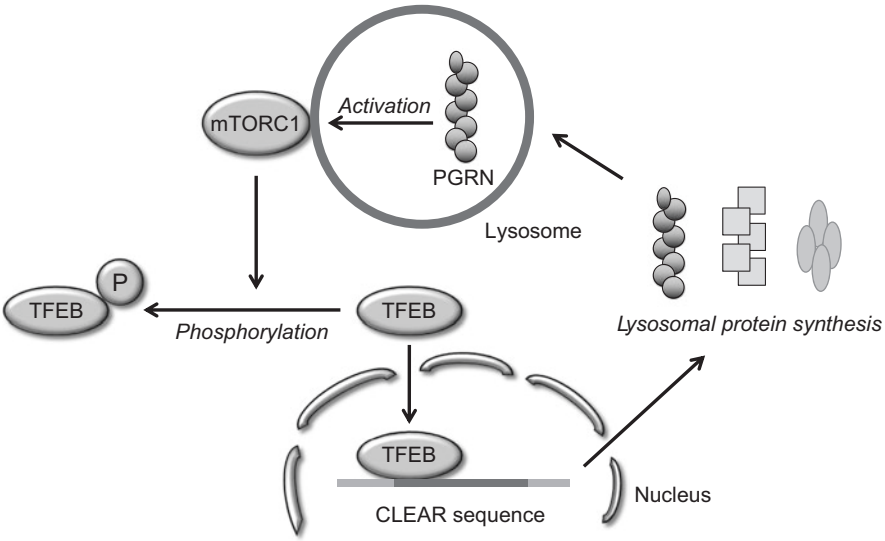

Fig. 2 PGRN in negative feedback loop of lysosomal biogenesis
The nuclear-transported TFEB, a master regulator of lysosomal genes, binds to the CLEAR sequence of DNA and activates transcription of genes for lysosomal biogenesis. Synthesized lysosomal proteins consist of lysosomes where PGRN results in activation of mTORC1. Activated mTORC1 phosphorylates TFEB, which inhibits nuclear-translocation of TFEB

expression was controlled by TFEB as well as other lysosomal gene expressions (Sardiello et al. 2009). While mTORC1-independent modulators such as suberoylanilide hydroxamic acid (SAHA) and trehalose increased PGRN levels, autophagy-lysosomal modulators through mTORC1-dependent pathway such as Torin1, rapamycin, bafilomycin A1 and chloroquine increased PGRN levels as well (Capell et al. 2011; Cenik et al. 2011; Holler et al. 2016; Tanaka et al. 2017). Therefore, the pathway through mTORC1 and TFEB partly regulates PGRN gene expression, which negatively regulates lysosomal biogenesis (Fig. 2).

Transcriptomics and proteomics studies confirmed not only a critical role of PGRN in lysosomal biogenesis, but also the regulation of innate immunity and lipid metabolism. Analysis of different age and regions of PGRN wild-type, heterozygous, deficient mice brain revealed an age-dependent up-regulation of many lysosomal genes and innate immunity related genes such as CD68, triggering receptor expressed on myeloid cells 2 (Trem2), and complement genes C1qa, b, c and C3 (Lui et al. 2016). The analysis using genome-wide RNA sequencing and microarray dataset detected a cluster with TYROBP forming a major hub that is composed of Trem2, one of the AD risk gene Ms4a7 (Karch et al. 2014; Chang et al. 2017), Cathepsin S and Z, complement system proteins, and Lyz2 (Takahashi et al. 2017). Lipidomic analysis suggested that dysfunction in lysosomal lipid metabolism might cause the specific changes of brain lipid composition such as triacylglyceride, diacylglyceride, phosphatidylethanolamine, and phosphatidylserine in FTLD resulting

from PGRN haploinsufficiency. Further, transcriptomic analysis using PGRN wild-type, heterozygous and deficient mice brains showed a number of differentially expressed transcripts involved in lipid metabolism (Evers et al. 2017). These results suggest that PGRN regulates innate immunity and lipid metabolism pathway in addition to lysosomal function. On the other hand, they might indicate possibility that lysosomal dysfunction from PGRN deficiency leads to the dysregulation of innate immunity and lipid metabolism pathway. Further studies are needed to work out the mechanistic connection between PGRN and these pathways.

PGRN Functions in Lysosomes

As mentioned above, there are increasing evidences that PGRN has a functional role in lysosomes. We previously focused on the relationship between PGRN and mature Cathepsin D (CTSDmat) levels, because the CTSDmat level is dependent on lysosomal function (Gieselmann et al. 1985) and FTLD patients resulting from PGRN showed increased CTSDmat levels as well as aged PGRN-deficient mice (Gotzl et al. 2014). We showed that PGRN down-regulation and deficiency increase CTSDmat levels as shown in the previous study, and exogenous PGRN partially rescued CTSDmat levels in the PGRN-deficient primary microglia (Tanaka et al. 2017). Then, PGRN overexpression decreased CTSDmat levels at least through its degradation by Cathepsin B (CTSB) that changes the target pH-dependently (Khouri

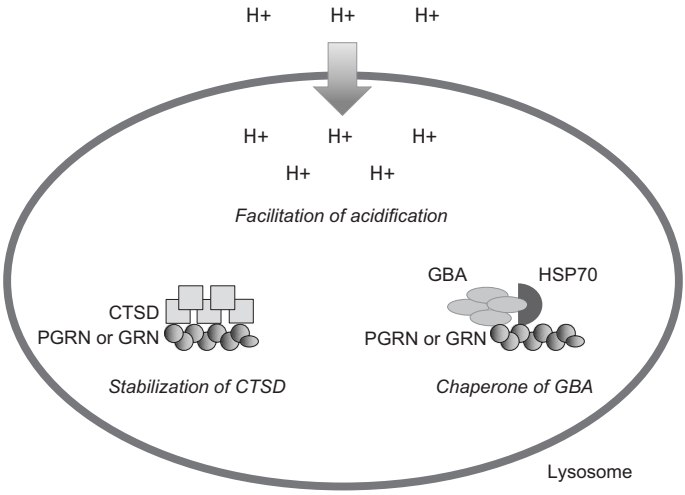

Fig. 3 PGRN functions in lysosomes
PGRN is involved in the regulation of lysosomal functions. (1) PGRN facilitates lysosomal acidification. (2) PGRN or granulin peptides (GRN) stabilizes CTSD. (3) PGRN works as a chaperone of GBA by connecting HSP70 to GBA.

et al. 1991). Remarkably, the action of PGRN expression to down-regulate lysosomal protein and gene expression levels was opposite to the effect of lysosomal alkalizers. More importantly, PGRN overexpression as well as the addition of secreted PGRN facilitated lysosomal acidification (Fig. 3). Furthermore, sortilin and CI-M6PR, endocytic receptors regulating lysosomal trafficking, mediated this effect, indicating that secreted PGRN is transported, and acidify lysosomes (Tanaka et al. 2017). Considering that the regulators of the gene expressions associated with autophagy-lysosomal pathway control the PGRN expression (Holler et al. 2016), PGRN might be a secretory lysosomal protein that regulates lysosomal function and biogenesis through the acidification of lysosomes. Lysosomal acidification is an essential process for the digestive function and to drive efflux of digested materials (Mindell 2012). An increase in the pH of lysosomal organelles prevents the secretory behaviour of acidic vesicles and leads to accumulation of acidic vesicles within the cell cytoplasm (Luciani et al. 2004). Actually, PGRN deficiency develops marked accumulation of lipofuscin, an end-product of lysosomal digestion (Ahmed et al. 2010; Wils et al. 2012; Tanaka et al. 2014; Valdez et al. 2017). Moreover, null mutations in *GRN* greatly reduce the number of released exosomes and alter their composition (Benussi et al. 2016). These findings support that PGRN deficiency suppresses digestion in and efflux from lysosomes. Interestingly, we showed that astrocytes or oligodendrocyte in aged PGRN-deficient mice accumulates p62 aggregates, although PGRN expression in these cells was not detected by immunohistochemistry (Tanaka et al. 2014). This finding clearly shows a non-cell autonomous action of PGRN, and suggests that PGRN secreted from neurons or microglia is incorporated into various cells in the brain, and counteracts lysosomal dysfunction resulting from aging by facilitating the acidification of lysosomes (Fig. 4). On the other hand, the level of TMEM106B, a risk modifier of FTLD-TDP of *GRN* mutation carriers (Van Deerlin et al. 2010), increases in the post-mortem brains of FTLD-TDP patients, and overexpression of TMEM106B inhibits lysosomal acidification (Chen-Plotkin et al. 2012). Interestingly, loss of TMEM106B partially ameliorated the pathological phenotypes associated with PGRN deficiency. In double knockout mice, loss of TMEM106B rescued a part of phenotype from PGRN deficiency such as retinal degeneration and hyperactivity (Klein et al. 2017), suggesting that PGRN and TMEM106B have opposite function in lysosome, and the decrease of PGRN and increase of TMEM106B in FTLD patients from PGRN haploinsufficiency contribute to the NCL-like phenotype. The mechanism regulating lysosomal acidification mediated by PGRN and TMEM106B should be useful to understand the pathology of FTLD.

PGRN is constituted of 7.5 GRN repeats which is processed into 6-kDa GRN peptides (Cenik et al. 2012). This is similar to PSAP that is synthesized as a precursor protein. PSAP is secreted, and transported into lysosomes by sortilin, and processed into 8–11 kDa saposin A, B, C, and D peptides, and function as a sphingolipid activator protein. Remarkably, PSAP or an individual saposin deficiency cause lysosomal storage diseases (Schulze and Sandhoff 2014). Therefore, it was previously predicted that GRN peptides have a function as an activator of lysosomal enzyme (Cenik et al. 2012). Recently, several groups showed that the inhibiton of lysosomal function increase the level of PGRN, suggesting that PGRN could be proteolytic

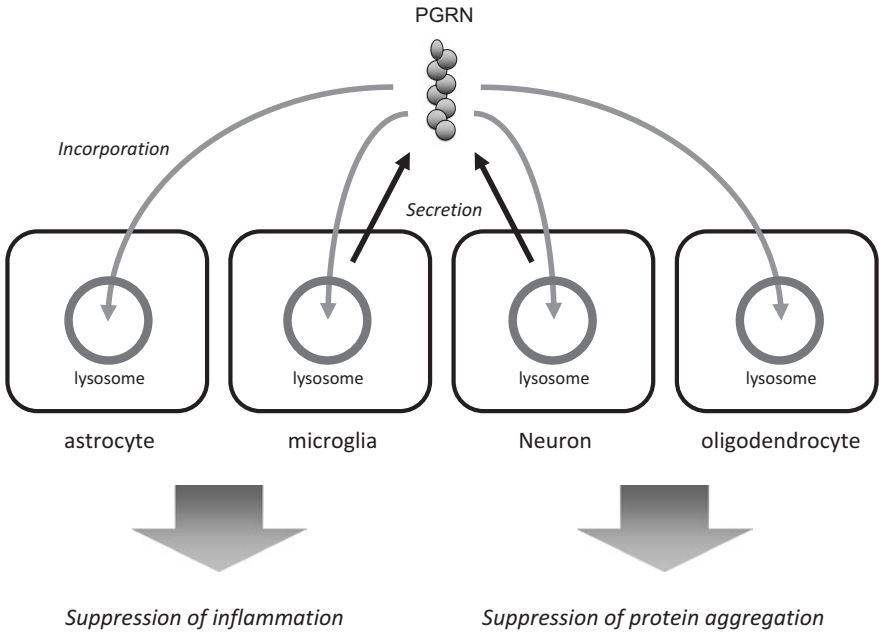

Fig. 4 The role of PGRN in cells composing the brain
PGRN is secreted from microglia and neurons in the brain. Secreted PGRN is incorporated and transported into lysosomes in neurons and glial cells. PGRN suppresses the formation of toxic protein aggregates in neurons and oligodendrocyte by facilitating lysosomal function. While it remains unknown about the mechanism, PGRN is suppressive to exacerbated inflammatory responses of astrocyte and microglia

processed in lysosomes (Tanaka et al. 2017; Holler et al. 2017; Lee et al. 2017; Zhou et al. 2017b). In fact, we found for the first time that the down-regulation and inhibition of a lysozyme Cathepsin B (CTSB) increases PGRN levels (Tanaka et al. 2017). On the other hand, the following studies clearly showed that Cathepsin L (CTSL) mediates the processing of PGRN into GRN peptides in lysosomes (Holler et al. 2017, Lee et al. 2017, Zhou et al. 2017b). Moreover, Lee et al. identified the CTSL cleavage sites within PGRN using liquid chromatography-mass spectrometry. Most of them reside within the linker regions and did not overlap those of neutrophil elastase (Lee et al. 2017). Surprisingly, GRN peptides were not detected in the media (Holler et al. 2017; Zhou et al. 2017b), suggesting that GRN peptides work only in lysosomes. To support this idea, several groups detected the precipitation of Cathepsin D (CTSD) by PGRN (Valdez et al. 2017; Zhou et al. 2017a) as well as the pulldown of CTSD by recombinant PGRN or GRN E peptide added to mouse brain lysate (Beel et al. 2017). Interestingly, these groups also showed the functional role of PGRN on the CTSD activity. The CTSD activity in the liver and spleen of 2-month-old PGRN-deficient mice is lower than wild-type mice (Zhou et al. 2017a). iPSC-derived heterozygous PGRN mutant neurons decrease the CTSD activity after 100 days following differentiation (Valdez et al. 2017). Moreover, Beel et al. showed

that PGRN stabilizes the matured CTSD independent of its activity (Fig. 3), and this function is mediated by GRN E peptide (Beel et al. 2017; Valdez et al. 2017). These findings suggest that CTSD turnover is faster in the neurodegenerative diseases resulting from PGRN insufficiency. CTSD deficiency cause NCL as well as PGRN deficiency (Carcel-Trullols et al. 2015), and their pathology overlap at least in mice (Gotzl et al. 2014). While CTSD levels increase along with lysosomal biogenesis resultant from PGRN insufficiency as mentioned above, lysosomal enzymes could regulate their levels each other (Barry and Platt 2012). Therefore, the decreased activity of CTSD in the PGRN-decreased condition might contribute the pathology in PGRN-associated diseases.

Apart from the relationship between PGRN and CTSD, a group identified beta-glucocerebrosidase (GBA) as another interacting partner of PGRN (Jian et al. 2016a). GBA is transcriptated from *GBA1* gene, and cleaves glucocerebroside into glucose and ceramide. An autosomal recessive mutation of *GBA1* gene causes Gaucher disease (GD) which is classified into lysosomal storage diseases, and *GBA1* mutation is also a strong risk factor for Parkinson's disease (PD) (Pitcairn et al. 2019). While there has been no report about connection between GBA and FTLD, they found a significant decrease in serum PGRN levels in GD patients compared to healthy controls, suggesting a possible association between PGRN and GBA. Moreover, they found that PGRN-deficient mice developed phenotypes characteristic of GD such as hepatosplenomegaly and glycolipid accumulated Gaucher-like cells (Jian et al. 2016b). Importantly, they found that GRN E peptide mediates the binding between GBA and heat shock protein 70 (HSP70), and loss of these binding leads to GBA aggregation in the cytoplasm in PGRN-deficient mice (Jian et al. 2016a). These findings indicate that PGRN functions as a chaperone for GBA through the binding of HSP70 (Fig. 3), suggesting that the possibility of GRN peptides as a chaperone for other proteins in lysosomes.

Conclusion

PGRN is a multifunctional protein involved in multiple physiological and pathological events. Most importantly, PGRN insufficiency causes incurable neurodegenerative diseases including FTLD-TDP and NCL. While the mechanism PGRN deficiency causes neurodegenerative diseases is still controversial, evidences that PGRN regulates lysosomal function and biogenesis are accumulating. The *GRN* gene has two possible CLEAR sequences in the promoter region where TFEB, a master regulator of lysosomal gene expression, binds, and thereby PGRN expression increases. PGRN directly or indirectly binds to endocytic receptors that transport lysosomal proteins into lysosomes such as sortilin or CI-M6PR. PGRN localizes to lysosomes and suppresses exacerbated lysosomal biogenesis at least through the acidification of lysosome. Aged PGRN-deficient mice exhibit selective vulnerability in the VPM/VPL region of thalamus, as do model mice of lysosomal diseases. GRN peptides stabilize CTSD and work as a chaperone of GBA.

Lysosomes are multifunctional organelles involved in not only degradation and recycling of cellular waste, but also secretion, plasma membrane repair, signalling, and energy metabolism. Therefore, PGRN deficiency might affect a broad range of physiologic and pathologic events. However, as mentioned above, PGRN has a dominant role in lysosomal homeostasis. Medications targeting the recovery of lysosomal function seem the most reasonable way for symptomatic improvement in FTLD-TDP or NCL patients resulting from PGRN insufficiency. While it still remains unknown how PGRN insufficiency leads to TDP-43 aggregation predominantly, these investigations about PGRN regulation of lysosomes highlight the importance of lysosomal function in the pathogenesis of neurodegenerative diseases.

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Progranulin in Sexual Differentiation of the Developing Brain

Masatoshi Suzuki

Abstract During the perinatal period (or known as the critical period), sex-dependent differentiation of the brain occurs in response to sex steroids. Steroid hormone exposure (androgen and estrogen) can induce masculinization during the critical period, otherwise the brain develops as feminine by default. Our previous studies indicated that progranulin (PGRN) gene would be involved in masculinization of developing brain in rats. In the neonatal rat hypothalamus, administering androgen and estrogen significantly increased expression of PGRN mRNA. The level of PGRN mRNA expression remained high in males throughout the critical period of sexual differentiation in the brain; however, in females PGRN mRNA expression gradually decreased. We detected high levels of PGRN mRNA in the ventromedial hypothalamic and arcuate nuclei of the hypothalamus. Next, we designed complementary antisense oligodeoxynucleotides to the PGRN mRNA sequence and injected them into the third ventricle of newborn male rats. After sexual maturation, the treated rats displayed significantly lower scores than the controls in a variety of tests assessing copulatory behavior. Interestingly, PGRN-deficient mice also exhibited a decrease in a specific parameter of male sexual behavior. Altogether, our studies demonstrate that PGRN critically works for the organization of the neuronal system that controls male-specific functions in the developing brain.

Keywords Brain · Estrogen · Neurogenesis · Progranulin · Sexual dimorphism

Introduction

A growth modulatory protein Progranulin (PGRN) has been known to have multifunctional roles in neurodegenerative disorders and normal brain development. PGRN is released by a multitude of cells, is a glycosylated protein, and is a potential

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trigger for mitosis in cell culture (Bateman and Bennett 1998). Extracellular proteases can split PGRN into several granulin peptides, which likely have differing functions. Initially identified as peptides of approximately 6-kDa, some granulin peptides are capable of modulating the growth of cells in culture (Bateman and Bennett 1998). Many tissues and organs have been identified to highly express PGRN mRNA, including the reproductive organs, neural tissues, gastrointestinal tract, and endocrinal organs (Bhandari et al. 1993; Daniel et al. 2000).

The potential role of PGRN in the central nervous system (CNS) has attracted a decent amount of attention. Specifically, recent studies indicate that PGRN critically works for disease progression in neurodegenerative diseases (Ahmed et al. 2007; Baker et al. 2006; Cruts et al. 2006; Brouwers et al. 2008; Sleegers et al. 2008). Mutations in the PGRN gene have been reported as the cause of frontotemporal lobar degeneration (FTLD) (Baker et al. 2006; Cruts et al. 2006), which is characterized by massive degeneration in the frontal lobe and temporal lobe of the brain. Furthermore, FTLD is recognized as the common cause of dementia after Alzheimer's disease (AD). Aside from loss-of-function mutations in FTLD, potential links of PGRN have been proposed in a number of neurodegenerative diseases in which microglial activation occurs. The diseases include AD, lysosomal storage-deficient disorders, Creutzfeldt-Jakob disease, viral encephalitis, and motor neuron diseases such as Amyotrophic Lateral Sclerosis (ALS) (Ahmed et al. 2007). PGRN has also been shown to be involved in neurotrophic activity and neuroinflammation in the CNS (Ahmed et al. 2007; Eriksen and Mackenzie 2008).

While biological roles of PGRN still need to be explored, in the meantime we have proposed a specific role of this molecule as a sex steroid mediator during brain development (Suzuki and Nishihara 2002; Suzuki et al. 1998, 2001, 2009). Throughout this chapter, we summarize our previous works and recapitulate the existing knowledge surrounding the potential roles of PGRN as a sex steroid mediator in sexual differentiation of the rat brain during perinatal period.

Sex Steroids and Sexual Differentiation of the Developing Brain

Sexual differences have been characterized in anatomical and functional aspects commonly in mammals (Arnold and Gorski 1984; McCarthy 2008; Negri-Cesi et al. 2008). These differences are quite obvious in reproductive functions by influencing hormone levels and behaviors in the reproductive system. Hormones and neurotransmitters show different levels between the sexes, but degrees of sex variation have also been identified in non-sexual functions such as spatial orientation and verbal fluency, or adaptive mechanisms of the adrenal axis to stress (McCarthy and Konkle 2005). Interestingly, a number of studies indicated that these differences are often independent of genetic sex.

The mechanisms seem to be conserved throughout mammalian species for the organization of sexual differentiation in the brain, although there may be some spe-

cies differences. During brain development, a male sex hormone, androgen, plays critical roles in the organization of neural circuits responsible for sexual dimorphic functions. These neuronal circuits control neuroendocrine, behavioral, and cognitive functions with sex-dependent manners (Arnold and Gorski 1984; MacLusky and Naftolin 1981). The organization of neural circuits by androgen occurs during the specific developmental period known as the critical period. Sexual differentiation in the male brain (*i.e.* masculinization of the brain) is primarily induced by testicular androgen.

The mechanisms of sexual differentiation, including the timing and length of the critical period during development, are somewhat modified in some species. In rats, testicular androgen still plays a major role in sexual differentiation of the brain but an additional mechanism is required. In the brain, androgen needs to be metabolized into estrogen by an enzyme, aromatase, when it works to induce masculinization of the brain (McEwen et al. 1977). Indeed, high levels of aromatase are identified in the specific brain nuclei that anatomically and functionally demonstrate sexually dimorphism (McEwen et al. 1977; MacLusky et al. 1987). In female rats, the brain develops an essentially female phenotype in the absence of circulating androgen. Although developing ovaries produce estrogen in the serum, it does not have influence in the female brain. High levels of α-fetoprotein, a protein that tightly bonds to estrogen, are identified in developing fetuses. Serum estrogen in females binds to α-fetoprotein, which prevents the effects of estrogen in the female brain. Interestingly in rodents, administration of sex steroids or their inhibitors can disturb (or reverse) the organization of brain circuits that are originally defined by genetic sex. Androgen injection in perinatal female rats can permanently masculinize specific behaviors and neuroendocrine functions. High estrogen dosage can also induce masculinization of males and females, and seems to mirror the effects of endogenous androgen. During the critical period of sexual differentiation in the rat brain, any disturbance of the endogenous hormonal balance by environmental influences (*i.e.* exposure to endocrine disrupting chemicals) causes in anatomical and functional abnormalities of sexual differentiation in the specific brain regions (Dohler 1998).

Identification of PGRN as a Sex Steroid-Inducible Gene

Sex steroids exert profound and selective influences on brain development via controlling transcription and/or translation of genes that would potentially be responsible for neuronal and glial cell differentiation (Arnold and Gorski 1984; MacLusky and Naftolin 1981; McEwen et al. 1977). Most of the biological effects of sex steroids occur through interactions with steroid receptors, which can work as transcriptional factors for a variety of downstream genes. The concept that transcriptional regulation via sex steroids is critical for the structural and functional sexual differentiation of the brain. This concept is supported by an early study using nucleoplasmic RNA polymerase inhibitors (Stanley et al. 1986). This led us to generating a new hypothesis that by analyzing the specific gene expression or

protein synthesis induced by sex steroids. We can efficiently come to understand the mechanism behind the sexual differentiation of the brain (Yonehara et al. 2002a, b, 2003).

In order to explore this hypothesis, we thought to pinpoint genes with contrasting expression between sexes or expression triggered by steroid treatment in the neonatal rat hypothalamus (Fig. 1) (Suzuki et al. 1998). We used an efficient method named the cDNA subtraction method to isolate clones that are disproportionately distributed between two different samples of cells or tissues (Tanaka et al. 1992). At day 2 post-birth (day 0 = the day of birth), female rats were subcutaneously injected with androgen (testosterone propionate, TP; 1 mg). This treatment has been shown to dysregulate the estrous cycle and render it constant in all the treated rats when they matured (Barraclough 1961). In a separate cohort of female pups, sesame oil was injected as a control. At day 5, all hypothalamic tissues were collected and used to prepare the cDNA libraries. The cDNA sequences from two sample groups were mixed, denatured, and hybridized in the reaction solution. Single-strand DNA sequences were isolated by hydroxylapatite chromatography and amplified by

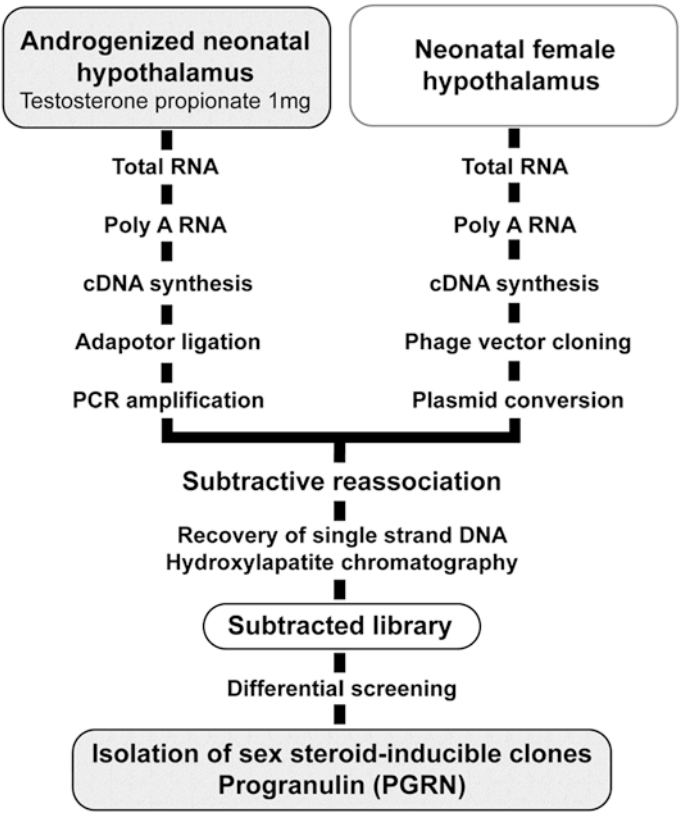

Fig. 1 Schematic illustration of the procedure for cDNA subtraction. (Suzuki et al. 1998, 2009)

PCR. The amplified PCR products were cloned to the phage vector and produced a subtracted cDNA library. Differential hybridization was then performed using duplicate filters. These filters were prepared from the subtracted cDNA library with the subtracted cDNA probe and the control hypothalamus cDNA probe. After the screening of 6×10^5 clones from the subtracted library, 52 clones were selected with a strong hybridization signal to the subtracted probe. The sequencing of these clones revealed that 32 of the 52 clones were partial homologs of the PGRN gene (Bateman and Bennett 1998; Shoyab et al. 1990; Bhandari et al. 1993). This result indicated that PGRN mRNA expression was specifically increased by androgen treatment in the neonatal rat hypothalamus.

Changes and Localization of PGRN mRNA in the Neonatal Rat Brain

To confirm the result of cDNA subtraction, PGRN mRNA level in the hypothalamus was determined by Northern blot using the isolated DNA fragment as a probe (Fig. 2) (Suzuki et al. 1998). PGRN gene expression in 5-day-old pups was significantly high in the hypothalamus of both male and androgenized (TP-treated) female when compared to the control female. Further, we compared PGRN mRNA expression in the hypothalamus between intact males and females during the perinatal period. The level of PGRN mRNA is maintained at high levels throughout the critical period in the hypothalamus of male rats. In contrast, PGRN mRNA expression was gradually decreased in females after birth.

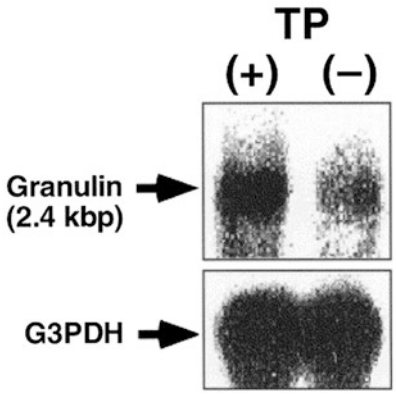

Fig. 2 Effect of androgen treatment on PGRN gene in the neonatal rat hypothalamus. Two-day-old female rats were treated with either 1 mg of testosterone propionate (TP) or its vehicle. Each group of hypothalami was collected at 5 days of age, and mRNA was extracted and used for the Northern hybridization to detect the expression of PGRN or internal control gene (glyceraldehydes-3-phosphate dehydrogenase; G3PDH). (This figure is reproduced from Suzuki et al. 1998; Suzuki and Nishihara 2002)

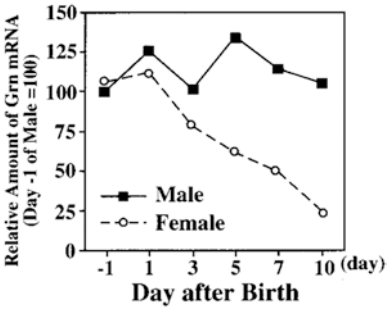

Fig. 3 Changes in PGRN mRNA expression levels in the hypothalamus of intact male and female rats during the perinatal period. Each value was normalized using the value of internal control gene (glyceraldehydes-3-phosphate dehydrogenase; G3PDH), relative to the defining value of 100 in males 1 day before birth. (This figure is reproduced from Suzuki et al. 1998); Suzuki and Nishihara 2002)

Next, we determined PGRN mRNA localization in the brain of our rats by *in situ* hybridization (Fig. 3) (Suzuki et al. 1998). In the male rat brain, PGRN mRNA was highly expressed in the specific nuclei of the hypothalamus: ventromedial hypothalamic (VMH) and arcuate nuclei. Interestingly, these hypothalamic nuclei are known as the brain areas where sex steroids affect the synaptic structures with a dense assembly of estrogen receptors. Furthermore, VMH has been known to serve the dimorphism of sexual behavior in rats.

Intraventricular Injection of PGRN Antisense Oligodeoxynucleotide in Neonatal Male Rats

Based on different patterns of PGRN gene expression in males and females, we hypothesized that high levels of PGRN expression in the neonatal hypothalamus critically impacts masculinization of the brain. To explore our hypothesis, we used the antisense oligodeoxynucleotides (ODNs) to block translation of the selective mRNA to protein (Suzuki et al. 2000). To achieve sufficient deliver of antisense ODNs into the cells, we used engineered liposomes incorporated with inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) (Yamada et al. 1996; Matsuo et al. 2000). We used HVJ-liposome antisense ODN complexes to specifically inhibit PGRN expression in the neonatal brain, and we then tested sexual behavior after the animals got matured.. At 2 days of age, we injected male rats with a PGRN complementary antisense ODN conjugated with HVJ-liposome (injection site at third ventricle). Upon maturation, the antisense ODN-treated animals had compromised male sexual behaviors as adults (Suzuki et al. 2000).

We tested copulatory behaviors of males without prior sexual experience for 30 minutes using estrogen-primed progesterone-injected ovariectomized females.

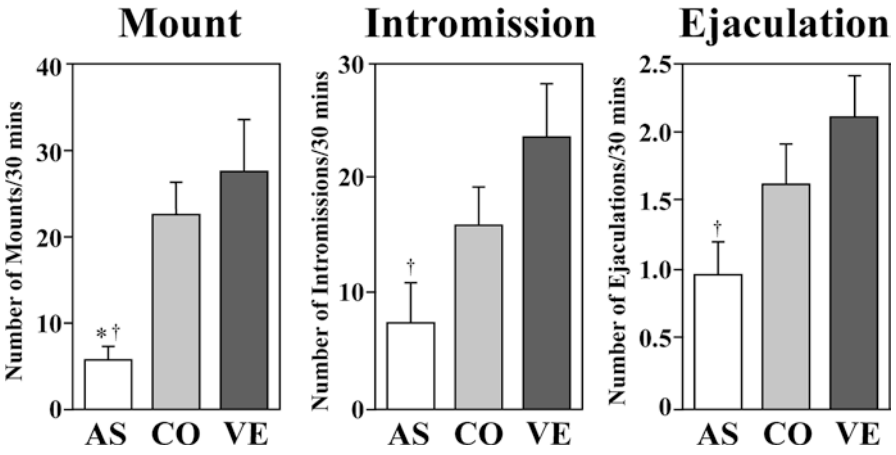

Fig. 4 Aberration of male sexual behaviors in male rats treated neonatally with the antisense oligodeoxynucleotide (ODN) complementary to PGRN mRNA sequence. Each column and vertical bar represent the mean ± SEM (n = 18 for AS, 12 for CO, and 11 for VE). When the male ejaculated during the 30 min of the experimental period, the number of mounts and intromissions from the point of female introduction to the first ejaculation was converted into the number per 30 min. AS, antisense ODN; CO, control ODN; VE, vehicle (HVJ-liposome). *: $P < 0.05$ vs CO; †: $P < 0.05$ vs VE. (This figure is reproduced from Suzuki et al. 2000, Suzuki and Nishihara 2002)

The antisense ODN treated rats showed a decrease in copulatory behaviors, especially that of ejaculation and post-ejaculation mounting; although, the difference did not reach significance. Frequencies of specific male behaviors, such as mounting, intromission, and ejaculation, were significantly decreased in the antisense ODN-treated group compared to the vehicle-treated group (Fig. 4). The frequency of mounting in the antisense ODN-treated group was also significantly lower than in the control-ODN group. Interestingly, the serum concentrations of reproductive hormones (testosterone and luteinizing hormones) were not affected by the antisense ODN treatment at the neonatal stage. Altogether, these results indicate that the organization of the male-specific neuronal network requires a sustained PGRN mRNA expression at high levels in the mediobasal hypothalamus during the critical period.

Sexual Dimorphic Behaviors in PGRN Knockout Mice

Next, we investigated male sexual behavior, aggression and anxiety in a line of mice with targeted disruption of the PGRN gene (Kayasuga et al. 2007). Gene knockout animals would work as an alternative tool to study the biological roles of PGRN *in vivo.* PGRN-deficient mice displayed a decreased level of ejaculation frequency. In contrast, the delay and occurrence of both mounting and intromission were unchanged. When the resident-intruder paradigm was used to test hostility, PGRN-deficient mice showed an increase in hostility. We found decreased levels of anxiety in wild-type

male mice compared to females by the open field test. Elevated anxiety levels were identified in PGRN-deficient males, although sex differences in anxiety were not displayed. Interestingly, gene expression of the serotonergic receptor 5-HT1A was decreased in the hippocampus of PGRN-deficient mice after aggressive encounters, which is potentially related to aggression and anxiety inhibition. Conversely, PGRN gene deficiency did not influence serum testosterone levels. These studies using PGRN-deficient mice show that PGRN plays at least a partial role in establishing sexual dimorphic behaviors through modulating the brain serotonergic system.

PGRN and Endocrine Disruptors

Endocrine disruptors, also known as environmental endocrine-disrupting chemicals (EDCs), are exogenous chemical substances that act like endogenous hormones and interrupt their biological functions in the endocrine system (Waldron and Naber 1974). EDCs are potentially harmful effects in animals, because low-level or long-term exposure of these substrates may cause comparable effects in humans. Specifically, the malleability and the high reactivity are high in the fetal brain. If the exposure to ECDs occurs during the critical period of brain sexual differentiation, it might cause adverse effects on reproductive functions (Dohler 1998; Suzuki et al. 2004).

Following our observations of the steroid-dependent induction and sexually dimorphic expression patterns, we hypothesized that the PGRN expression could work as a sensitive indicator for evaluating sex steroid properties of EDCs in the neonatal brain. To test this hypothesis, we determined how the perinatal exposure of some phthalate/adipate esters influences PGRN mRNA level in the neonatal hypothalamus and sexual behaviors after maturation (Fig. 5). Phthalate/adipate esters have been known as types of EDCs that potentially disturb the endocrine system, Perinatal exposure to these chemicals significantly affected PGRN expression in the hypothalamic tissues of male and female rats (Fig. 5). Additionally, the exposure to these chemical compounds during the critical period averted sexual behaviors at maturity. Our results indicate that perinatal exposure to ECDs may influence PGRN gene expression in the hypothalamus of neonatal rats, which leads permanent effects on the hypothalamus and changes the exhibition of sexual behaviors after maturation.

Conclusion

Increased levels of sex steroids during the critical period significantly increased PGRN gene expression in the rat brain. The studies using antisense-ODNs and PGRN-knockout mice indicate that PGRN expression critically works for the organization of the brain system to manifest sexual dimorphic behaviors. PGRN expression would work as a good indicator to evaluate the estrogenic or anti-estrogenic effects of EDCs.

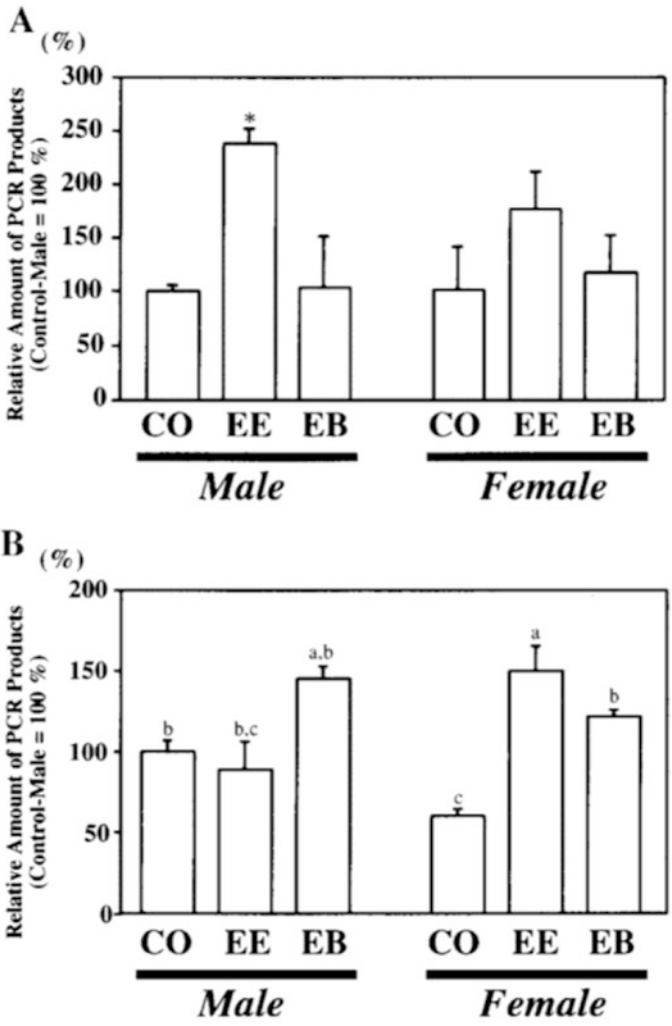

Fig. 5 Increased expression of PGRN mRNA by in the hypothalamus of 6-day-old (A) and 10-day-old (B) rats. Two different estrogens were used, ethinyl estradiol (EE) or estradiol benzoate (EB). EE was orally administered to dams from 15 days of gestation to the day of sampling, while EB was subcutaneously injected into the pups at day 2 (day 0 = the day of birth). Each group of hypothalami was collected, and mRNA was extracted and used for semi-competitive RT-PCR using PGRN or G3PDH primers. Each value was normalized using G3PDH values and is presented relatively, with the values in the control males defined as 100%. *: $P < 0.05$ vs. other groups in A. In B, values with the same letter are not significantly different ($P > 0.05$). (This figure is reproduced from (Suzuki et al. 2001, Suzuki and Nishihara 2002)

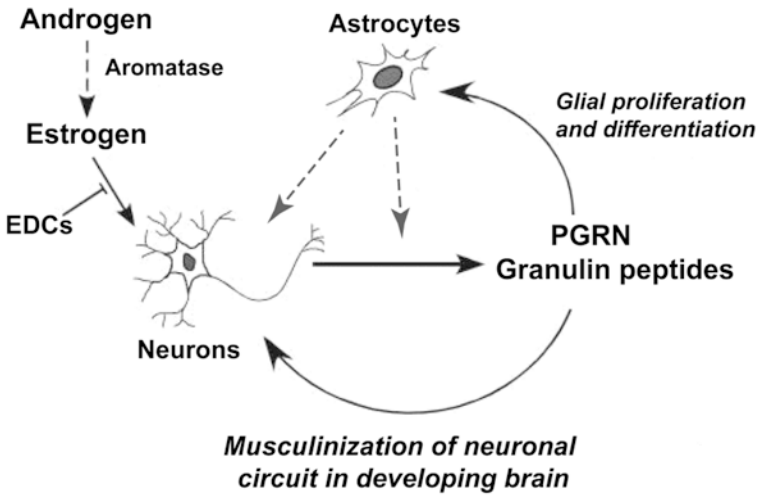

Fig. 6 Proposed roles of PGRN for sexual differentiation of the rat brain. Estrogen metabolized from androgen by aromatase increases PGRN expression in neurons. The secreted PGRN or cleaved granulin (GRN) peptides modulates the proliferation and differentiation of neurons and/or glial cells in an autocrine and/or paracrine manner, and consequently masculinizes the neuronal circuit in developing brain. Exposure to endocrine disruptors (EDCs) may lead inappropriate expression of PGRN gene. Astrocytes may contribute to control the levels of PGRN and GRN peptides. (This figure is modified from Suzuki et al. 2009)

These observations support our fundamental hypothesis: PGRN is involved in the organization of the male brain during the critical period. Although specific roles of PGRN in the CNS development remain unknown, PGRN may modulate the proliferation and/or differentiation of neurons and/or glial cells in an autocrine or paracrine manner. Consequently, PGRN may contribute to formulate the neuronal circuit required for exhibiting sexual dimorphic behaviors (Fig. 6). Further studies are valuable to elucidate how sex steroids modulate PGRN expression in the developing CNS.

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Progranulin and Inflammation/ Neuroinflammation

Masato Hosokawa

Abstract Progranulin (PGRN) and granulin (GRN) peptides are involved in peripheral inflammatory diseases and neuroinflammation. PGRN was shown to have an anti-inflammatory effect in its role as an antagonist of the TNF-α signaling pathway. On the other hand, GRN peptides stimulate immune cells to secrete pro-inflammatory cytokines. PGRN reduction worsens peripheral inflammatory disorders such as inflammatory bowel disease, skin inflammation and acute lung or kidney injury. In the brain, loss of PGRN induced an inflammatory state in head injury, ischemia or neurodegenerative diseases. Interestingly, microglia isolated from a *granulin (Grn)*-deficient mouse was reported to express much of pro-inflammatory cytokines. *Grn*-deficient microglia may have a tendency toward inducing a hyperactive pro-inflammatory state when activated, and this hyperactivation may contribute to neuronal cell death. Since overexpression of PGRN, bone marrow transplantation from wild type mice or supplementation with recombinant PGRN reversed this inflammatory state, these approaches may have therapeutic potential for treating peripheral inflammatory diseases and neurodegenerative diseases caused by *GRN* deficiencies.

Keywords GRN peptides · Microglia · Neuroinflammation · Pro-inflammatory cytokines · Tumor necrosis factor (TNF)-α

Introduction

Neuroinflammation is observed in neurodegenerative diseases such as Alzheimer's disease and frontotemporal lobar degeneration. The involvement of innate immune system, including microglia and the complement proteins, is seen. However, the

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classical four major symptoms of inflammation (flare, fever, swelling and pain) do not occurre in neuroinflammation. It is thought that neuroinflammation depends on factors made in the brain. Recently, progranulin (PGRN) and its degradation products, granulin (GRN) peptides, have been reported to be involved in neuroinflammation. This section mainly reviews the biological function of PGRN in inflammation and neuroinflammation.

PGRN and Inflammation

Li et al. reported that cytokines regulated PGRN expression. Pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, enhanced *GRN* gene expression in murine embryo fibroblasts (Li et al. 2002). The promoter regions of mouse and human *GRN* gene had regulatory elements that were involved in cytokine expression. (Baba et al. 1993; Bhandari et al. 1996)

PGRN bound directly to secretory leukocyte protease inhibitor (SLPI) and was protected from cleavage by elastase (Zhu et al. 2002). SLPI is produced by macrophages and neutrophils and is known to inhibit the inflammatory response (Jin et al. 1997). During inflammation, elastase was released by neutrophils and acted on PGRN to produce GRN peptides by cleaving linker regions (Zhu et al. 2002). GRN-B peptide stimulates epithelial cells to secrete the pro-inflammatory cytokine, IL-8 which is a major chemoattractant for neutrophils and monocytes, but PGRN has no such effect. PGRN suppressed the TNF-α induced respiratory burst of neutrophils, but GRN-A and GRN-B peptide did not show such inhibitory effect (Zhu et al. 2002). These results suggested pro-inflammatory and anti-inflammatory roles for GRN peptide and PGRN, respectively.

PGRN and TNF-α Signaling Pathway

Tang et al. reported that PGRN bound directly to tumor necrosis factor receptors (TNFRs) and antagonized TNF-α signaling (Tang et al. 2011). Their co-immunoprecipitation (Co-IP) experiment showed that PGRN interacted with TNFR2 in chondrocytes. Recombinant human PGRN bound to the extracellular domains of TNFR1 and TNFR2 in a dose-dependent manner. They used a collagen-induced arthritis mouse model and found that administration of PGRN reversed the severe inflammatory arthritis in *Grn*-deficient mice. PGRN could inhibit TNF-α-mediated activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling. Jian et al. extended Tang's discovery; they showed that PGRN directly bound to the cysteine-rich domains 2 and 3 of the TNFR extracellular domains (Jian et al. 2013). They suggested that the anti-inflammatory role of PGRN was mediated through direct inhibition of TNFR1. PGRN inhibited the expression of TNF-α-inducible genes such as C-X-C motif chemokine Ligand 9 (CXCL9), CXCL10,

intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in murine bone marrow-derived macrophages (BMDMs) and the human cell line, THP-1 (Mundra et al. 2016; Tian et al. 2014).

On the other hand, Chen et al. rebutted these phenomena. They performed Co-IP using recombinant PGRN (rPGRN) and recombinant TNFR1 or TNFR2. rPGRN did not interact with the two TNFRs (Chen et al. 2013). They extended the study and also performed surface plasmon resonance (SPR) studies. rPGRN did not bind TNFR1 or TNFR2. In this SPR studies, PGRN could bind to sortilin which was reported as a PGRN receptor (Hu et al. 2010). However, only the last three amino acids (QLL) of PGRN were required for interacton to sortilin (Zheng et al. 2011). It was pointed out that sortilin was not a suitable positive control for TNFR by Wang et al. (Wang et al. 2015). Addition of rPGRN could not directly antagonize TNF-α-induced neurotoxicity in dopaminergic neuron-like cells and lipopolysaccharide (LPS)-induced signaling in a microglia cell line. Their study suggested that neuroinflammation is not caused by disruption of PGRN-TNFR interactions. Another group supported these data: PGRN did not inhibit TNF-α signaling through TNFR1 (Etemadi et al. 2013). The PGRN-TNFR interaction had been a controversial issue, but a proper selection of chip for a SPR assay resolved the problem. Using an appropriate selection of chip for a SPR assay demonstrated that PGRN bound to TNFR (Jian et al. 2013; Wang et al. 2015).

PGRN and Peripheral Inflammation Related to Diseases

PGRN upregulation was observed in inflammatory bowel diseases (IBD), not only in dextran sulfate sodium (DSS)-induced or picrylsulfonic acid (2, 4, 6-trinitrobenzenesulfonic acid, TNBS)-induced colitis models, but also in colon tissues from human IBD patients (Wei et al. 2014). *Grn*$^{-/-}$ mice were highly susceptible to DSS- and TNBS-induced colitis compared to WT mice. Interestingly, rPGRN administration attenuates the inflammatoty responses in DSS-induced colitis mice dramatically. This PGRN-mediated protection against chemically-induced colitis required IL-10 signaling. PGRN could not alleviate intestinal inflammation in the DSS-induced colitis model mice when using TNFR2-deficient mice (Wei et al. 2014).

PGRN is involved in the pathogenesis of skin inflammation. PGRN expression level was increased in oxazolone (OXA)-induced dermatitis in mice (Zhao et al. 2013). *Grn*$^{-/-}$ mice showed more severe inflammation induced by OXA skin than did WT mice. The mRNA levels of inflammatory markers, including IL-1β, IL-6, cyclooxygenase-2 (COX-2) and induced nitric oxide synthase (iNOS), were significantly increased in OXA-induced *Grn*$^{-/-}$ mice. Atsttrin, a derivative of PGRN, which is composed of three TNFR-binding domains of PGRN (Tang et al. 2011), effectively attenuated OXA-induced inflammation (Zhao et al. 2013). Huang and colleagues reported similar outcomes using the 12-O-tetradecanoyl-phorbol 13-acetate (TPA)-induced psoriasis-like inflammation mouse model (Huang et al.

2015). PGRN expression is upregulated in both psoriasis vulgaris skin lesions and in the psoriasis-like mouse skin lesions induced by TPA. *Grn*$^{-/-}$ mice exhibited more severe psoriasis-like inflammation when treated TPA, and showed from reduced regulatory T cells in the cervical lymph nodes (Huang et al. 2015).

Using LPS-induced acute lung injury (ALI) mouse model, PGRN administration downregulated the pulmonary inflammation with reduced number of neutrophils, pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and chemokines (Guo et al. 2012). In this study, PGRN-TNFR2 interaction was shown to be important for the protective role of PGRN. Similar results were reported from another research group (Yu et al. 2016).

In an LPS-induced acute kidney injury (AKI) mouse model, PGRN expression levels were augmented in WT mice and *Grn*$^{-/-}$ mice were particularly susceptible to LPS-induced AKI (Xu et al. 2016). Administration of rPGRN before LPS-injection attenuated renal cell death, production of pro-inflammatory cytokines and inflammation-related reactions in endotoxin-induced AKI in WT mice.

Total joint arthroplasty (TJA) is performed for the treatment of severe joint diseases, including rheumatoid arthritis and osteoarthritis. Aseptic loosening, which is characterized by chronic inflammation, occurs as a result of the biological response to such wear debris as titanium (Ti) particles. Zhao et al. found that Ti particles elicited PGRN expression in murine macrophage-like cells (RAW264.7), and in a mouse air-pouch model of inflammation (Zhao et al. 2016). In this model, *Grn*$^{-/-}$ mice exhibited enhanced mRNA and protein expression of IL-1β, IL-6 and TNF-α compared those with WT mice. Administration of rPGRN reversed Ti particle-induced inflammation; mRNA and protein expression levels of IL-1β, IL-6 and TNF-α were reduced compared with vehicle (phosphate-buffered saline) treated mice. PGRN also suppressed Ti-induced osteolysis ex vivo and in vivo in mice. Mechanistic studies indicated that PGRN reduced the Ti particle-induced TNF-α-NF-κB signaling (Zhao et al. 2016).

PGRN and Neuroinflammation

Yin et al. revealed that *Grn*$^{-/-}$ macrophages produced more pro-inflammatory cytokines and less anti-inflammatory IL-10 than WT macrophages (Yin et al. 2010a). They isolated BMDMs from *Grn*$^{-/-}$ or WT mice stimulated with the Toll-like receptor (TLR) 4 agonist, LPS. *Grn*$^{-/-}$ and WT BMDMs showed similar cell-surface marker expression and phagocytic ability. However, *Grn*$^{-/-}$ BMDMs produced higher levels of such mRNA and proteins as monocyte chemoattractant protein-1 (MCP-1), CXCL1, IL-6, IL-12p40 and TNF-α. By contrast, expression of the anti-inflammatory cytokine IL-10 was greatly reduced in *Grn*$^{-/-}$ BMDMs at both of transcript and protein levels (Yin et al. 2010a). In the *Grn*$^{-/-}$ brain, upregulation of astrocytes and microglia was observed in age-dependent manner. These are the sign of dysregulated inflammatory response, named gliosis. Such age-dependent gliosis was also reported by other groups (Ahmed et al. 2010; Ghoshal et al. 2012; Petkau et al. 2012; Wils et al. 2012; Yin et al. 2010b).

To investigate the biological function of PGRN, rat primary cortical neurons were treated with PGRN. Secretion of Th2 cytokines such as IL-10, IL-4 and IL-5 and of chemokines, such as interferon-inducible protein-10, MCP-1, matrix metalloproteinase-13 (MMP-13), etc., was detected in PGRN-treated primary neurons, while classical pro-inflammatory cytokines were not upregulated (Pickford et al. 2011).

Bossu et al. assessed circulating levels of pro-inflammatory cytokines in FTLD patients with and without *GRN* loss-of-function mutation, serum IL-6 levels in FTLD patients with the *GRN* mutation were significantly increased as compared to FTLD patients without the *GRN* mutation (Bossu et al. 2011). Serum levels of TNF-α and IL-18 did not differ significantly between the two groups.

Experimental Mouse Models and Neuroinflammation

PGRN-deficient *($Grn^{-/-}$)* mice showed neuroinflammation and neuronal cell death following toxin-induced damage (Martens et al. 2012). The acute brain injury model was produced using 1-methyl-4-(2′-methylphenyl)-1, 2, 3, 6-tetrahydrophine (MPTP) which is a neurotoxin targeting the dopaminergic neurons of the substantia nigra. $Grn^{-/-}$ mice brains showed more neuronal cell loss and increased microgliosis when compared with WT mouse brains (Martens et al. 2012). Primary cell cultures of this model, showed there were no differences in the numbers of surviving tyrosine hydroxylase and TUJ1 (β tubulin 3) positive neurons in the WT or $Grn^{-/-}$ mice. However, $Grn^{-/-}$ microglia expressed neurotoxic factors that induced neuronal cell death. Martens and colleagues showed that $Grn^{-/-}$ microglia expressed much more of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 compared with WT microglia treated with LPS/IFN-γ. Moreover, IL-10 mRNA expression was decreased in $Grn^{-/-}$ microglia after LPS/IFN-γ treatment, although IL-10 protein expression was increased in the media, reflecting differences in the regulation of mRNA and protein. These data suggested that $Grn^{-/-}$ microglia had a tendency toward a hyperactive pro-inflammatory condition when activated, and this hyperactivation may contributes to the neuronal cell death (Martens et al. 2012).

Glial cells isolated from PGRN-overexpressing transgenic mice showed neuroprotective effects against LPS-induced totoxicity. The expression of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α was decreased in the glial cells of PGRN-overexpressing Tg mice compared with those of wild type mice following middle cerebral artery occlusion (MCAO) (Tao et al. 2012). The level of the anti-inflammatory cytokine, IL-10, was increased in the glial cells from PGRN-overexpressing Tg mice (Tao et al. 2012). Neuroinflammation is thought to be a main contributor of brain injury after cerebral ischemia. Using MCAO mice model, Egashira and colleagues demonstrated that PGRN was involved in neuroinflammation following cerebral ischemia (Egashira et al. 2013). In this MCAO model, PGRN expression was reduced, whereas PGRN administration attenuated the infarct volume and brain swelling. They also revealed that PGRN inhibited TNF-α binding to the neutrophil surface and suppressed the neutrophil chemotaxis induced by TNF-α in dose-dependent manner (Egashira et al. 2013).

Using a mouse experimental traumatic brain injury (TBI) model, Tanaka and colleagues elucidated the relationship between PGRN and neuroinflammation (Tanaka et al. 2013a). PGRN was mainly produced from CD68-positive microglia and downregulated hyper-neuroinflammatory reaction after TBI in mice. PGRN deficiency *(Grn$^{-/-}$)* did not affect the injury size, but it increased CD68-positive microglia after TBI. Furthermore, *Grn$^{-/-}$* showed upregulated levels of transforming growth factor β1 (TGF-β1) and phosphorylated Smad3 (pSmad3) which is downstream of TGF-β1 signaling. Additionally, pSmad3 was increased in astrocytes on the injured side. They suggested that the excessive TGF-β1-Smad3 signaling pathway caused by PGRN deficiency might contribute to the progression of glial scar formation (Tanaka et al. 2013a). In this TBI mice model, the expression of lysosome-related genes was significantly increased in *Grn$^{-/-}$* mice compared with WT mice (Tanaka et al. 2013b). The precise mechanism of this phenomenon is explained in another section of this book.

Homozygous *Grn* knock-out *(Grn$^{-/-}$)* mice have been used as a frontotemporal dementia (FTD) model and exhibit social deficits and neuroinflammation with microglial activation. Homozygous *GRN* mutations leading to complete PGRN protein insufficiency were defined as neuronal ceroid lipofuscinosis. It was found that the complete lack of PGRN protein *(GRN$^{-/-}$)* may have influences distinct from those of haploinsufficiency *(GRN$^{+/-}$)*. Filiano et al. found that *Grn$^{+/-}$* mice showed age-dependent social deficits similar to FTD, but different from *Grn$^{-/-}$* mice, since there were no gliosis or upregulation of TNF-α in *Grn$^{+/-}$* mice (Filiano et al. 2013). These results indicated that FTD-related behavioral deficits resulting from PGRN haploinsufficiency might occur even in the absense of detectable gliosis and neuroinflammation.

Using the *Grn$^{-/-}$* mice and experimental TBI model, Menzel and colleagues investigated the relationship between TBI and astrogliosis (Menzel et al. 2017). *Grn$^{-/-}$* mice showed exaggerated astrogliosis 5 days after experimental TBI and astrocytes in *Grn$^{-/-}$* mice at perilesional sites were immunopositive for iNOS and TNF-α. Primary astrocytes were isolated from WT mouse brain and cultured with LPS or TNF-α in the presence of murine rPGRN. The rPGRN reduced LPS- and TNF-α-induced expression of iNOS and TNF-α mRNA. Furthermore, intracerebroventricular injection of rPGRN immediately before experimental TBI downregulated brain damage and neurological deficits (Menzel et al. 2017). These results suggested that PGRN might attenuate pro-inflammatory activation of astrocytes *in vitro* and i*n vivo.*

PGRN Prevents Neuroinflammation

To prevent neuroinflammation in *Grn$^{-/-}$* mice, Yang and colleagues performed WT bone marrow transplantation (BMT) (Yang et al. 2014). The BMT via retroorbital venous plexus injection 1 day after total body irradiation partially reconstituted PGRN in the periphery and in the cerebral cortex of *Grn$^{-/-}$* mice. A pro-inflammatory

condition in vivo and ex vivo preparations of cerebral cortex of *Grn*$^{-/-}$ mice could be partially to fully reversed by a BMT. BMT will be a potential treatment for neurodegenerative diseases caused by *GRN* deficiencies.

There is a change of PGRN in the brain after subarachnoid hemorrhage (SAH) and it plays a role in early brain injury, Zhou and colleagues studied the expression levels of PGRN and pro-inflammatory cytokines in cerebrospinal fluid (CSF) from SAH patients and from experimental SAH rat (Zhou et al. 2015). The expression levels of PGRN were significantly reduced and the levels of myeloperoxidase, IL-1β and TNF-α were remarkably increased in the CSF from SAH patients. PGRN levels in the brain also decreased after experimental SAH in rats and administration of rPGRN reduce brain water content and improved neurological scores at 24 hrs after experimental SAH. These changes were suggested that the expression of pro-inflammatory cytokines was decreased in the brain (Zhou et al. 2015).

PGRN Involvement in Neurogenesis and Neuroinflammation

Ma et al. evaluated the involvement of PGRN in neurogenesis and neuroinflammation in the hippocampus of mouse brain (Ma et al. 2017b). Using an LPS-induced immune stress model, they revealed that LPS-stimulus induced upregulation of PGRN in activated microglia and decreased neurogenesis in the dentate gyrus of the hippocampus. PGRN deficiency *(Grn*$^{-/-}$*)* enhanced the expression of pro-inflammatory gene such as IL-1β, TNF-α, IL-6 and microsomal prostaglandin E synthase-1 after LPS treatment. *Grn*$^{-/-}$ also increased the suppressive effects of LPS on hippocampal neurogenesis. These results demonstrated that PGRN might facilitate hippocampal neurogenesis and abrogate excessive neuroinflammatory reactions after LPS treatment (Ma et al. 2017b). Hippocampal neurogenesis is decreased in aged mice, and there was no significant difference between WT mice and *Grn*$^{-/-}$ mice (Ma et al. 2017a). The expression of pro-inflammatory genes was upregulated with age, particularly in *Grn*$^{-/-}$ mice. These results suggested that, especially in aged mice, *Grn*$^{-/-}$ increased neuroinflammation by microglia, and the downregulation of hippocampal neurogenesis might not be offset by PGRN.

Summary

To summarize this chapter, the biological roles of PGRN and GRN peptides in neuroinflammation are shown in Fig. 1. PGRN induces anti-inflammatory conditions in the brain which leads to increased IL-10 and reduced IL-1β, IL-6 and TNF-α. Total loss of PGRN or degradation from PGRN to GRN peptides by elastase elicits neuroinflammation and increases pro-inflammatory cytokines, leading to gliosis in the brain. Pro-inflammatory cytokines enhance *GRN* gene expression in microglia.

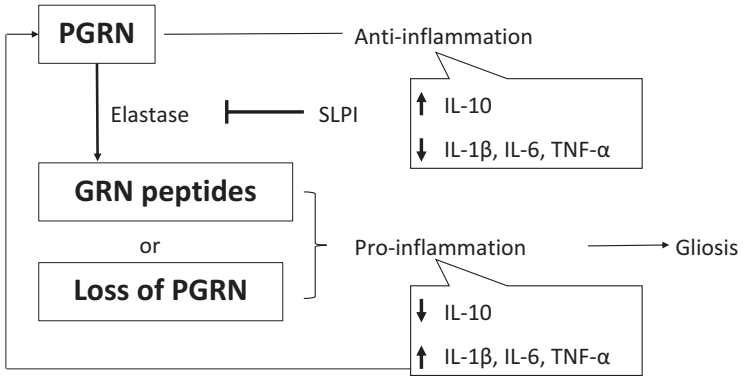

Fig. 1 Schematic diagram of "feedback loop" of PGRN and inflammation/neuroinflammation

PGRN and inflammation constitute a feedback loop. Inhibition of PGRN degradation by SLPI or supplementation of rPGRN or Atsttrin, a derivertive of PGRN may have therapeutic potential for preventing inflammatory reactions related to diseases.

PGRN induces release of IL-10 and reduces pro-inflammatory cytokines. Loss of PGRN or GRN peptides degraded by elastase prompts immune cells to secrete pro-inflammatory cytokines. The released pro-inflammatory cytokines augment PGRN production in microglia.

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Neural Stem/Progenitor Cells and Progranulin

Taku Nedachi

Abstract Progranulin (PGRN) is widely expressed in the body, including the central nervous system (CNS), and controls cell growth, proliferation, differentiation, cell death, *etc*. In this chapter, I discuss recent findings about the roles of PGRN in neurogenesis, with a focus on its roles in neural stem and progenitor cells (NSPCs). Accumulated evidence clearly suggests that PGRN engages in neurogenesis; however, the precise mechanisms remain largely elusive. One reason is that many different cells in the CNS produce PGRN and can also react to PGRN. This produces a complex challenge to understand PGRN-dependent neurogenesis *in vivo*. Therefore, I initially introduce recent studies that analyze the roles of PGRN in neurogenesis. Although details about PGRN-dependent signaling in NSPCs are widely unknown, recent studies describe the physiological receptors for PGRN. The reported PGRN receptors also have binding capacities for other factors; therefore, I discuss the potential interaction between PGRN and other growth factors that control the cellular fates of NSPCs. Moreover, levels of PGRN potentially control distribution of the extracellular matrix in the CNS; conversely, the abundance of the extracellular matrix may modulate PGRN action. Therefore, the relationship between PGRN and the microenvironment, or niche, which plays a crucial role in determining the NSPC fates, is also discussed. Overall, recent studies gradually suggest that PGRN directly or indirectly influences NSPCs; however, further experiments are required to unveil the precise contribution of PGRN to neurogenesis.

Keywords Progranulin · Neurogenesis · NSPCs · Signaling · Extracellular matrix

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Introduction

A century ago, Santiago Cajal was the first to describe the morphological diversity of neurons in the brain. Since then, significant effort has been made to study how the structure and function of neural circuits are generated and maintained. In addition, many scientists strive to contribute to our understanding of complex behaviors as well as many diseases caused by the destruction of neural circuits. Most of these complex organisms are maintained by the balance of genesis and death of cells; therefore, one of the long-discussed questions in this field is whether the timing of "neurogenesis" was limited to developmental stages or could it be observed in adulthood. In other words, is the loss of neurons in adult mammals reversible?

A significant contribution to answer this question was made in 1962. Altman (1962) used intracranial injection of thymidine-H^3 and demonstrated that the proliferation of neurons could be observed in adult rats. Moreover, Eriksson et al. (1998) showed the presence of neural progenitor cells in the human dentate gyrus (DG), which might explain why neuroplasticity is observed not only in rodents but also in humans. These and other elegant studies have supplied converging evidence that mammalian adult neurogenesis occurs in two distinct regions of the brain: the subgranular zone (SGZ) of the DG of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles, although the latter has been confirmed in rodents but not in humans. In these regions, the neural stem and progenitor cells (NSPCs) are the origin of the neurons, astrocytes, and oligodendrocytes.

Proliferation and differentiation of NSPCs are the most important features for neurogenesis. These events produce both neuronal and glial progeny to compensate for the loss of cells in the central nervous system (CNS). Neural stem cells (NSCs) have self-renewal capacity and can differentiate into three cell lineages: neurons, astrocytes, and oligodendrocytes (Park et al. 2012). Regulation of NSPCs is tightly controlled by multiple factors. Several studies have demonstrated that neurogenesis is regulated by behavioral experiences (Gould and Tanapat 1999), exercise (van Praag et al. 1999), and environmental enrichment (Kempermann et al. 1997). The new neurons mature and integrate into existing neuronal circuits (van Praag et al. 2002). This maturation and integration is extremely important to maintain brain functions such as learning and memory (e.g., Winocur et al. 2006; Saxe et al. 2006; Dupret et al. 2008; Imayoshi et al. 2008; Deng et al. 2009; for a review, see Marín-Burgin and Schinder 2012). Thus, neurogenesis appears to be directly engaged with not only neural plasticity but also learning and memory formation. In addition, an important question is how neurodegenerative diseases occur as a consequence of a disruption to this system.

The maintenance of proliferation and differentiation of NSPCs is regulated by the microenvironment, or "niche", which consists of at least three factors: (A) secreted soluble factors, (B) cell-cell contacts, and (C) the extracellular matrix (ECM). The purpose of this chapter is to introduce the role of progranulin (PGRN) in neurogenesis; therefore, I will mainly focus on describing PGRN as a secreted soluble factor. However, I will note that recent studies have demonstrated that these three factors (A-C) do not function independently but work in an orchestrated manner as described later.

PGRN in Neurogenesis

PGRN (also called granulin-epithelin precursor, PC-derived growth factor, acrogranin, or proepithelin) is a 67.5 kDa glycoprotein that consists of 593 amino acids. It contains seven distinctive repeated structures, called granulin motifs, which are enriched with cysteine residues. Generally, PGRN stimulates cell proliferation and attenuates cell death similar to other growth factors (He and Bateman 2003; Ong and Bateman 2003).

Since the discovery of the roles of PGRN in neonatal sexual differentiation of the brain and the development of frontotemporal lobar degeneration (FTLD) (Suzuki et al. 1998, 2000; Cruts et al. 2006; Baker et al. 2006), one crucial question is which types of cells secret PGRN in the CNS. Lü et al. (2013) investigated the expression of PGRN in NSCs and their differentiated cell lineages. This *in vitro* study clearly indicates that PGRN is highly expressed in NSCs obtained from the SVZ of neonatal Sprague Dawley rats and in their differentiated cell lineages. We also confirmed the expression and secretion of PGRN from cultured NSPCs obtained from the SGZ of prenatal C57BL6 mice (Nedachi et al. 2011). Thus, it seems that NSPCs secret PGRN *in vitro* and *in vivo*. In addition, the expression of PGRN has been observed in neurons, microglia, astrocytes and oligodendrocytes in the brain tissue of neonatal rats (Lü et al. 2013). Overall, this series of experiments strongly suggests that PGRN is expressed in multiple cell types in the CNS.

The precise roles of PGRN in NSPCs function are still largely under investigation. In NSPCs derived from wild type mice (WT-NSPCs) and PGRN-deficient mice (KO-NSPCs), exogenous PGRN treatment significantly enhances the proliferation of KO-NSPCs (Nedachi et al. 2011). In contrast, exogenous PGRN treatment does not alter NSPC death or asymmetrical cellular division, which results in the production of NSPCs, neurons, astrocytes, and oligodendrocytes. Thus, it seems that exogenous PGRN treatment affects NSPC proliferation rates, whereas its effects on NSPC death and differentiation rates are minimal. Intriguingly, our study also showed that in the absence of PGRN, KO-NSPCs display a higher cell death ratio than WT-NSPCs, even though exogenous PGRN treatment has no effect (Nedachi et al. 2011). This also raises the possibility that in NSPCs, intracellular PGRN that has never been released has some specific function.

Additional evidence of the involvement of PGRN in neurogenesis has been reported in a zebrafish model. Walsh and Hitchcock (2017) recently studied the effects of PGRN depletion on retinal neurogenesis. Zebrafish have progranulin-A and –B, which are related to human PGRN with 44.8% and 42.9% identity, respectively (Cadieux et al. 2005), and the depletion of progranulin-A results in a significant lengthening of the cell cycle. This delayed neurogenesis may contribute to a delay in retinal development. Moreover, Ma et al. (2017a) demonstrated that PGRN protects hippocampal neurogenesis via suppression of neuroinflammatory responses under acute immune stress. They first showed that treatment of mice with lipopolysaccharide (LPS) significantly increases the expression of PGRN in activated microglia and at the same time, decreases neurogenesis in hippocampus. Interestingly,

PGRN deficiency does not alter the age-related decreases in neurogenesis (assessed by counting Ki67-IR and DCX-IR cells), although this does still exacerbate microglial activation in the hippocampus (Ma et al. 2017b).

Overall, several discrepancies remain between *in vitro* and *in vivo* models. Increases in PGRN levels in the CNS do not directly engage in the enhancement of neurogenesis, and evidence suggests that PGRN controls neurogenesis in the CNS *via* both direct and indirect action. PGRN induction by microglial activation may be involved in the latter indirect mechanism.

PGRN Signaling in NSPCs

PGRN initiates the activation of signaling pathways, which are often triggered by other conventional growth factors. To be specific, PGRN frequently stimulates two major signaling molecules found in many types of cells: extracellular signal regulated kinase (ERK1/2) and phosphatidylinositol 3-kinase (PI3K) (Toh et al. 2011). He et al. (2003) demonstrated that PGRN stimulates the proliferation and migration of rat primary fibroblasts and endothelial cells, and ERK1/2 and PI3K activation are involved in this process. Similarly, PGRN-dependent activation of ERK1/2 and PI3K has been observed in embryonic fibroblasts derived from PGRN-deficient mice (Kleinberger et al. 2010). Moreover, in the 5637 bladder cancer cell line, PGRN-dependent ERK1/2 activation seems to be coupled with the formation of the paxillin/FAK/ERK complex (Monami et al. 2006). In Her2-overexpressing breast cancer cells, it has been reported that PGRN activates c-Src, which may be an upstream signaling molecule for ERK1/2 and PI3K (Kim et al. 2016). In mouse cortical neurons, PGRN appears to activate the PI3K pathway. Kleinberger et al. (2010) showed that PGRN treatment induces the phosphorylation of Akt, which is a major downstream target of PI3K; however, phosphorylation of ERK1/2 was not detected (Kleinberger et al. 2010). In addition, PGRN induces Ser9 phosphorylation of glycogen synthase kinase-3β (GSK-3β), a substrate for Akt in mouse cortical and hippocampal neurons (Gao et al. 2010). Overall, the PI3K and ERK1/2 pathways are major PGRN signaling pathways, although some differences are observed across cell types. Since PGRN is a multifunctional growth factor, these differences in signaling pathways may contribute to the selectivity of PGRN-dependent action across cell types.

Compared to the above information about PGRN signaling in different cell types, PGRN signaling in NSPCs remains widely elusive. However, we recently demonstrated that the administration of PGRN enhances GSK-3β phosphorylation (Ser9) in NSPCs derived from $Grn^{-/-}$ mouse hippocampus at embryonic day 16.5 (Nedachi et al. 2011). Adding LY294002, a PI3K inhibitor, abolishes this PGRN-dependent GSK-3β phosphorylation, which suggests that PGRN-dependent PI3K cascades are important for this GSK-3β phosphorylation. Moreover, we could not detect any PGRN-induced ERK1/2 activation (Nedachi et al. 2011), as other studies have done using mouse neurons (Kleinberger et al. 2010; Gao et al. 2010). Thus, although further studies are required, PGRN activates PI3K cascades in both NSPCs and

neurons; however, the impact on ERK1/2 activation seems to be minimal. Remarkably, the Ser9 phosphorylation of GSK-3β decreases its activity, and pharmacological inhibition of GSK-3β activity enhances neurogenesis in both humans and mice (Lange et al. 2011; Nedachi et al. 2011). Thus, PGRN-dependent neurogenesis may be mediated by this GSK-3β phosphorylation.

PGRN Shares Its Receptors with Other Factors in the CNS

Numerous factors are secreted by many cell types in the CNS. These factors originate in either the brain or other tissues/organs and can directly or indirectly modulate functions of NSPCs. Some of these factors may facilitate neurogenesis via control of PGRN action.

Microglia secret cytokines, which induce neurogenesis and oligodendrogenesis in the SVZ of the rat during the early postnatal days (Shigemoto-Mogami et al. 2014); however, the direct effects of activated microglia on NSPC survival are still controversial because of their spatial and temporal regulation. LPS treatment inhibits neurogenesis via the release of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) from microglia (Monje et al. 2003). TNF-α signaling is crucial for neurogenesis. Two types of TNF-α receptors, TNFR1 and TNFR2, are expressed in NSPCs. TNFR2 mainly transduces signals, which is important for basal neurogenesis; conversely, TNFR1 attenuates neurogenesis (Chen and Palmer 2013).

PGRN binds directly to TNFR1 and 2 (Tang et al. 2011); thus, one attractive hypothesis is that PGRN competitively binds to TNF-α receptors, thereby regulating neurogenesis. This PGRN-TNFR interaction was first identified in yeast two-hybrid screenings and was further confirmed using several other techniques, such as co-immunoprecipitation in human cells, the ELISA-based solid phase binding assay, and the surface plasmon resonance (SPR) approaches (Jian et al. 2013; Tang et al. 2011). Disrupting TNF signaling is a promising therapeutic target for many types of inflammatory diseases and conditions, therefore, the PGRN-TNFR interaction was extensively studied by other groups. Some groups successfully confirmed the PGRN-TNFR interaction (Alquézar et al. 2016; Liu et al. 2014; Thurner et al. 2015); however, Chen et al. (2013) claimed that they could not detect any PGRN-TNFR interaction in their antibody pull-down experiments using the Biacore system. Overall, it appears that PGRN and TNF-α may competitively facilitate the fates of NSPCs, although the PGRN-TNFR interaction is still controversial (Wang et al. 2015).

PGRN may also competitively act with neurotrophins (NTs) and pro-neurotrophins (pro-NTs) in NSPCs. NT signaling is crucial for maintaining the CNS (Chao 2003; Vilar and Mira 2016). The NT family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT4/5) (Bothwell 2014). Two distinct receptors were identified for NTs, Trk tyrosine kinases and p75 neurotrophin receptor (p75NTR) (Friedman and Greene 1999). p75NTR interacts with both NTs and pro-NTs, such as pro-NGF and pro-BDNF (Hempstead 2014). Remarkably, a Vps10 family pro-

tein, sortilin (Sort1), has the ability to initiate pro-NTs-induced cell death by forming a complex with p75NTR (Domeniconi et al. 2007; Jansen et al. 2007; Nykjaer et al. 2004; Teng et al. 2005). Strikingly, Sort1 induces rapid endocytosis of pro-NGF (Nykjaer et al. 2004), with little recycling (Nielsen et al. 2001). Therefore, NTs and pro-NTs seem to control the levels of Sort1 on the cell surface.

Recently, Sort1 was also identified as a receptor for PGRN (Hu et al. 2010). To the best of my knowledge, the expression of Sort1 in NSPCs has not been reported; however, Sort1 is abundantly expressed in both human embryonic stem cells (hESCs) and mature neurons (Lee et al. 2014; Sarret et al. 2003). Upon binding to Sort1, PGRN is endocytosed to lysosomes (Hu et al. 2010). This endocytosis appears to be important for determining extracellular PGRN levels, because ablation of the Sort1 gene fully normalizes PGRN levels in $Grn^{+/-}$ mice, which basally display PGRN haploinsufficiency (Hu et al. 2010). Expression patterns of NTs in the neurogenic niches of SVZ and SGZ have been extensively studied. For instance, NGF and BDNF are expressed in astrocytes, while NT-3 is expressed in ependymal cells (Vilar and Mira 2016; Tonchev 2011). Thus, even though further studies are required, especially to investigate the expression of Sort1 in NSPCs, Sort1 may have roles in the control of both NT and PGRN action via endocytosis and its expression on the membrane.

PGRN and the Extracellular Matrix

NSPCs are generally observed in the adjacent basal lamina of blood vessels which enrich the extracellular matrix (ECM) (Doetsch 2003). Although the precise roles are currently under investigation, ECM molecules seem to intervene in neurogenesis (Seki 2003; Mercier et al. 2002; Goetz et al. 2006; Kazanis and ffrench-Constant 2011). In this section, I focus on the relationship between the ECM and PGRN, which may be crucial for the cell fate determination of NSPCs.

Mercier et al. (2002, 2003) analyzed the adult neurogenic niche and found a series of "laminin"-immunoreactive puncta in the subependymal zone (SEZ). Laminins are heterotrimeric proteins containing one α-, one β-, and one γ-chain (Miner and Yurchenco 2004; Aumailley et al. 2005). Laminins have the capacity to bind to cell surface receptors such as integrins (Gullberg and Ekblom 1995) and exert multiple biological activities, including cell adhesion, cell migration, neurite outgrowth, and more (Kleinman et al. 1990; Ekblom et al. 2003; Ichikawa et al. 2009). Kazanis et al. (2010) explored the SEZ to identify the potential relationship between laminins and NSCs. They found that NSCs and precursors are surrounded by a laminin-rich ECM. However, importantly, NSCs express low levels of α6β1-integrin, a crucial membrane protein in the attachment of laminin, whereas precursors express higher levels of this protein. This laminin-integrin interaction is important for the control of proliferation activities in these cells, because *in vivo* blocking of β1 integrin induces the proliferation of precursors (Kazanis et al. 2010).

Interestingly, Tanaka et al. (2013) reported that PGRN deficiency exacerbates inflammatory responses related to activated microglia after experimental traumatic brain injury. They observed not only microglial activation but also the accumulation of laminins in the CNS. Overall, it could be hypothesized that the exacerbated inflammatory responses caused by PGRN deficiency enhance laminin accumulation in the CNS, followed by modulation of the fates of the precursor cells.

Another ECM molecule, collagen, is also observed in the neurogenic niche (Kerever et al. 2007). In the lateral ventricle walls, specific ECM structures defined as "fractones," which are enriched with the laminins, perlecan and collagens, have been observed (Kerever et al. 2007). Moreover, Mori et al. (2013) showed the migration of glial cells differentiated from NSPCs depends on the stiffness of the gel; the glial cells spread more widely on the stiff collagen gels compared to the less stiff collagen gels. Thus, the abundance of collagen may influence NSPC behavior. Very recently, we performed a transcriptome analysis comparing NSPCs obtained from wild-type and PGRN-deficient mouse hippocampus (manuscript in preparation). In this experiment, we found that the deficit of PGRN in NSPCs potentially enhanced the expression of some types of collagen family genes. Overall, PGRN appears to control NSPCs-derived collagen production, thereby potentially modulating the NSPCs niche.

In addition, some extracellular proteins have been reported to interact with PGRN in the other organs. For example, Guo et al. (2010) reported that PGRN binds directly to disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-7 and -12 in chondrocytes and inhibits degradation of cartilage oligomeric matrix protein (COMP) (Xu et al. 2007). In addition, PGRN has the capacity to bind to Perlecan, a heparan sulfate proteoglycan found in most basement membranes and cell surfaces (Gonzalez et al. 2003) and has been implicated in tissue development and several diseases (Arikawa-Hirasawa et al. 1999, 2001). Interestingly, Perlecan captures fibroblast growth factor (FGF)-2 and is crucial for FGF-2 signaling in the NSC niche (Kerever et al. 2007, 2014).

Although further studies are required to understand the relationship between PGRN and ECM in NSPCs, this information allows us to speculate that PGRN has the potential to modify the ECM surrounding NSPCs. At the same time, the modified ECM conditions may also affect PGRN action on NSPCs.

Conclusion

In conclusion, converging evidence suggests that the dynamics of NSPCs are controlled by PGRN via direct, cooperative or competitive action with other growth factors and/or ECMs (Fig. 1). However, given that different types of cells in the CNS produce PGRN and also react to it, and because PGRN promotes multiple biological actions, the precise impact of an excess or deficit of PGRN on NSPCs requires further investigation.

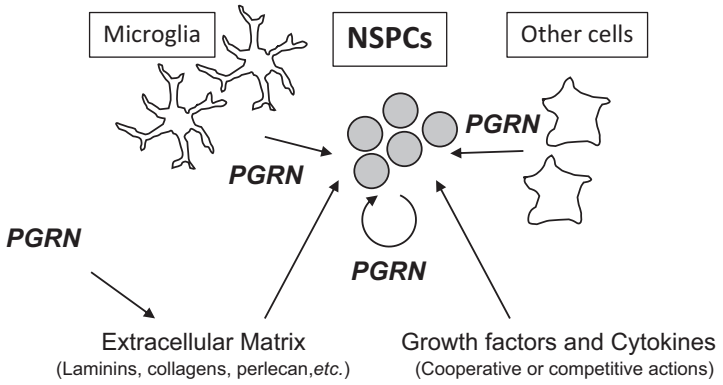

Fig. 1 The fates of neural stem/progenitor cells (NSPCs) are influenced by PGRN directly and/or indirectly

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Generation and Phenotyping of Progranulin-Deficient Mice

Takashi Matsuwaki

Abstract Progranulin (PGRN) is a multifunctional growth factor involved in many physiological and pathological processes in the brain. PGRN is expressed in a wide variety of tissues and organs including neural tissues, reproductive organs, endocrine organs, and gastrointestinal tract. We have previously reported that PGRN is one of the major factors involved in masculinization of the brain of rodents during neonatal period. To further evaluate the masculinizing role of PGRN, we have generated a line of PGRN-deficient mice. Male PGRN-deficient mice showed decreased ejaculation incidence and increased anxiety, implying the disrupted masculinization of the brain. We secondly focused on the PGRN function in the hippocampus and the cerebellum as those are the regions with high expression of PGRN in the brain. In the PGRN-deficient mice, the facilitative effect of voluntary exercise on adult hippocampal neurogenesis was blunted while the suppressive effect of immune challenge was exacerbated. Furthermore, PGRN-deficient mice showed a higher density of Purkinje cell dendrites in the molecular layer of the cerebellum, which possibly leads to the motor dysfunction we detected in those mice. In conclusion, we have demonstrated that PGRN functions to develop and maintain the neuronal circuits not only in the neonatal but also in the manured brain.

Keywords Progranulin · Masculinization · Anxiety · Hippocampus · Purkinje cells · Estrogen receptor α

Generation of PGRN KO Mice

We have previously determined progrnulin (PGRN) as one of the major factors involved in masculinization of the rodent brain (Suzuki et al. 2000). For further understanding of the mechanism of brain masculinization, we have generated a line

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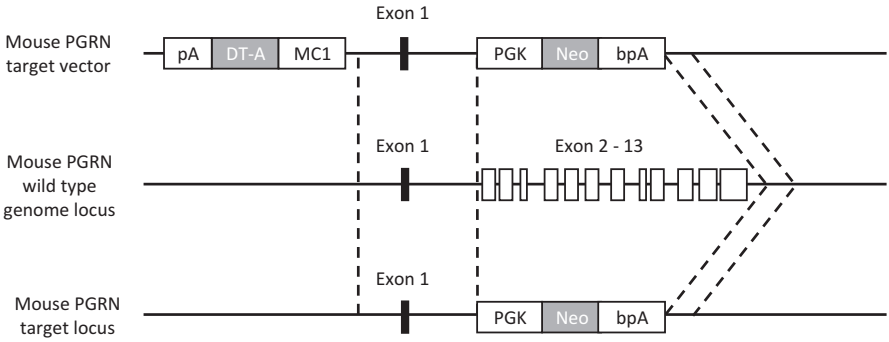

Fig. 1 The targeted strategy for the mouse genomic PGRN locus
Constructs of the mouse PGRN targeting vector (top), the wild-type mouse PGRN genome locus (middle) and the recombineted gene locus (bottom). In the mutant locus, the genomic region from exon 2 to exon 13 (appreoximatly 4.7 kb) was replaced by the PGK-Neo-bpA cassette

of mice with targeted disruption of PGRN gene (Kayasuga et al. 2007). In the mouse, the PGRN gene is approximately 6.3 kbp in length and consists of 13 exons (Baba et al. 1993). Granulin peptides of approximately 6 kDa are derived from PGRN (Bateman et al. 1990; Bhandari et al. 1992). The vector design for targeted strategy is shown in Fig. 1. First, we isolated a PGRN genomic clone from the genomic library of 129 SvJ mouse (Stratagene, La Jolla, CA). In the targeting vector, the 4.7 kb allele with exons 2–13 of the PGRN gene was replaced with a PGK-Neo-bpA cassette (Soriano et al. 1991) and a fragment of diphtheria toxin A (Yagi et al. 1993) was ligated to the 5′ end (Fig. 1). After linearization, the targeting vector was electroporated into E14.1 ES cells (Asano et al. 1997). For the generation of chimeric mice, ES cells were aggregated with two (C57BL/6 × DBF1) F1 eight cell stage embryos, according to the method described previously (Horai et al. 1998). Finally, one germ line chimera was obtained and his heterozygous offspring were backcrossed to C57BL/6 females. In total, we obtained 442 offspring by crossing heterozygous (HZ) male and female mice, whose genotypes were as follows; 28.1% (124/442) wild-type (WT), 49.3% (218/442) HZ and 22.6% (100/442) PGRN-deficient (KO). Both male and female of HZ are fertile and the litter size from HZ pairs (6.3 ± 0.3, n = 10) was comparable with that of WT pairs (7.9 ± 0.2, n = 18). In addition, there was no significant difference in the number of weaned pups at 30 days of ages between WT parents (6.9 ± 0.2, n = 15) and KO parents (4.7 ± 0.3, n = 9).

Alteration of Brain Masculinization in PGRN KO Mice

We have performed behavioral tests to examine possible impairment in the brain masculinization of PGRN KO mice. First, male-type sexual behavior was investigated using WT, HZ and KO mice at the age of 7–11 weeks. Mount, intromission

and ejaculation in 1 h were analyzed for three times with 4-day intervals. Throughout the three trials, both HZ and KO mice showed comparable latency and frequency of mount and intromission to those of WT mice. On the other hand, the ejaculation incidence was disrupted in KO mice. They displayed lower incidence of ejaculation at the second trial (Fig. 2a) and lower percentage of mice showing ejaculation at

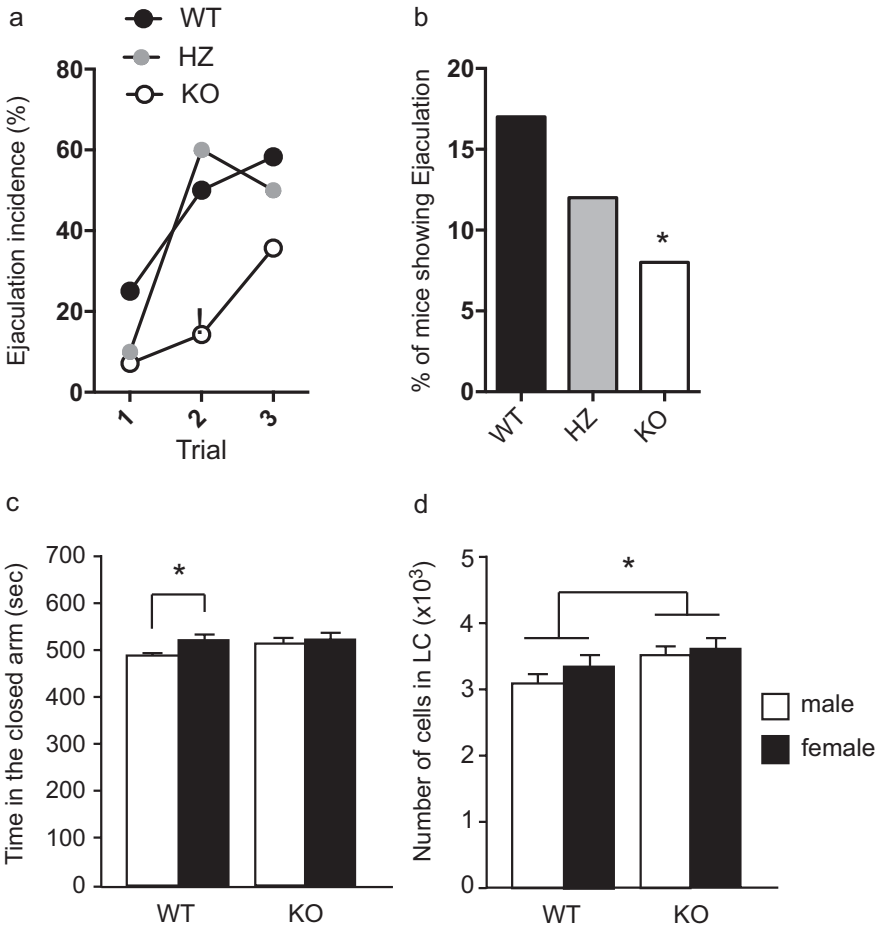

Fig. 2 Alteration of brain masculinization of male PGRN-deficient mice
(**a** and **b**) Percentage of mice showing ejaculation in each trial (**a**) and over the three trials (**b**). Each male was tested for 1 h with a stimulus female and behavior was recorded. The incidence of ejaculation was determined by the presence of vaginal plugs in females after the test. *p < 0.05, Fisher's exact probability test, compared to WT and HZ mice (**a**) and to WT mice (**b**) (n = 12 for WT, 10 for HZ, and 14 for KO). (**c**) The time within the closed arms analyzed in 10-min elevated plus maze test. Each column and vertical bar represent means ± SEM (n = 10 for male WT, 15 for female WT, 6 for male KO and 10 for female KO). *P < 0.05. (**d**) The number of cells in the LC in the male and female mice. Each column and vertical bar represent means ± SEM (n = 5 for all groups). *P < 0.05

least once over three trials (Fig. 2b), comparing with WT mice. This impairment of sexual behavior in the male PGRN KO mice is well correspondent with the results demonstrated in aromatase KO male mice (Bakker et al. 2004) and estrogen receptor (ER) KO (Wersinger et al. 1997) male mice, in which the lack of exposure to, or receptors for, estrogens during development impairs expression of male sexual behavior in adulthood. During the sexual behavior tests, aggressive behaviors toward females were noticed in KO male mice. WT male mice displayed a very few number of attacking toward female mice, which is well corresponding with the fact that this kind of aggressive behavior is quite unusual. However, KO mice showed a much larger number of aggressive bouts against the female than WT male mice, although it is not statistically significant. Serum concentration of testosterone did not differ among the males of the three genotypes, suggesting that PGRN does not affect the neuroendocrine system controlling gonadotropin secretion.

For further investigation of the influence of PGRN deletion on aggression, we next performed resident-intruder tests to evaluate the tendency of offensive intermale fighting of PGRN KO mice. The tests were repeated three times at 3-day intervals. The frequency of biting attacks was significantly higher in KO mice than that in WT and HZ mice. As it has been demonstrated that serotonergic neuronal system is related to inhibition of aggression, we then analyzed the hippocampal expression levels of the receptors for serotonin, 5-HT1A and 1B after three trials of resident-intruder test. The experience of aggressive encounters significantly decreased the mRNA expression of 5-HT1A in KO mice but not in WT mice or HZ mice. KO mice showed the tendency of decrease of 5-HT1B mRNA expression after aggressive encounters, although without significance. These changes in mRNA expression of serotonergic receptors in the hippocampus were well correlated with the changes in aggression behaviors in KO mice.

As aggressive behavior and anxiety is closely related to each other and elevated anxiety is often associated with an enhancement of aggression (Kikusui et al. 2004), we have evaluated anxiety tendency of KO mice using open field test (OFT) and elevated plus maze test (EPM). In OFT, male WT mice spent significantly less time in the peripheral area while longer time in the center than female WT mice. In EPM, there was a trend for the male WT mice to spend more time in the open arms than female WT mice. Moreover, male WT mice spent less time in the close arms. These results imply that in WT animals, male mice have less anxious tendency comparing to the female. The anxiety of male KO mice was enhanced to the similar level of the female and subsequently, the sexual dimorphism of anxiety has disappeared in KO mice (Fig. 2c). Taken together, the enhancement of aggressive behavior in male KO mice is possibly derived from elevated anxiety due to insufficient masculinization of the brain.

Subsequently, the morphologies of locus ceruleus (LC) and paraventicular nucleus (PVN) were observed to clarify the histological bases of sex difference in anxiety. Both PVN and LC are involved in anxiety and reported to have sexual dimorphism in their structure (Ishunina and Swaab 1999). The male WT mice showed a tendency to have a smaller volume and cell number of LC than WT mice. Volume and cell number of the LC were larger in KO mice comparing with WT mice (Fig. 2d), although no sex differences were detected in both genotypes of this

strain. Volumes of PVN were not significantly different between either genotypes or sexes. KO mice showed the comparable growth carve of body and brain weights with those of WT mice, implying that they have normal development of body and brain. During the maturation process of central nervous system, apoptosis in the LC starts from day 20 of pregnancy and reaches a peak at day 1 after birth, which is well coincident with the so-called critical period of sexual differentiation of the mammalian brain (Pinos et al. 2001). Thus, the volume and cell numbers of the LC is possibly under the effect of PGRN during the perinatal period. LC is the largest nucleus of noradrenergic neurons in the brain. Serotonergic neurons in the dorsal raphe project into the LC (Bremner et al. 1996), and secretion of noradrenalin is inhibited by a 5-HT1A agonist (Rioja et al. 2007), suggesting that these two monoaminergic systems are coordinated in the brain (Mongeau et al. 1997).

Deficiency of PGRN causes suppression of male-type sexual behavior, enhancement of anxiety and resultant aggressive behavior, which represents insufficient masculinization of the brain. Furthermore, PGRN probably plays a considerable role in organization of monoaminergic systems, which regulates anxiety in a sex-dependent manner.

PGRN Mediates the Enhancive Effect of Voluntary Exercise on Hippocampal Neurogenesis

Adult neurogenesis occurs predominantly in two regions, *i.e.* the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone lining the lateral ventricles (Chiba et al. 2009). Adult neurogenesis is upregulated by some growth factors, environmental enrichment, estrogen treatment and voluntary exercise, while downregulated by various stressors and high concentrations of glucocorticoids (Yin et al. 2010; Petkau et al. 2012). Our previous immunohistochemical study showed that PGRN is expressed broadly in the brain, with some strong expression in a certain regions, including the singulate and piriform cortices, the pyramidal cell layer and DG of the hippocampus, the amygdala, the ventromedial and arcuate nuclei of the hypothalamus, and the Purkinje cell layer in the cerebellum (Matsuwaki et al. 2011). Among them, hippocampus is one of the regions that have strong expression of PGRN. Thus, we have evaluated the possible role of PGRN in hippocampal neurogenesis of the adult brain.

We have placed a running wheel in the home cage of WT and KO mice to drive them to their voluntary exercise. The travel distance of KO mice was slightly but not significantly shorter than that of WT mice [WT mice, 9.93 ± 0.72 km/day (mean ± SEM) vs. KO mice, 8.00 ± 0.54 km/day], implying that KO mice have normal motor performance. Four-week voluntary exercise increased the expression of PGRN in the hippocampus of WT mice (Fig. 3a, b). Subsequently, we performed a double-labeling immunostaining for PGRN with neuronal nuclei (NeuN) as a neuron marker, ionized calcium-binding adapter molecule 1 (Iba-1) as a microglia marker, or glial fibrillary acidic protein (GFAP) as an astrocyte marker. It has been

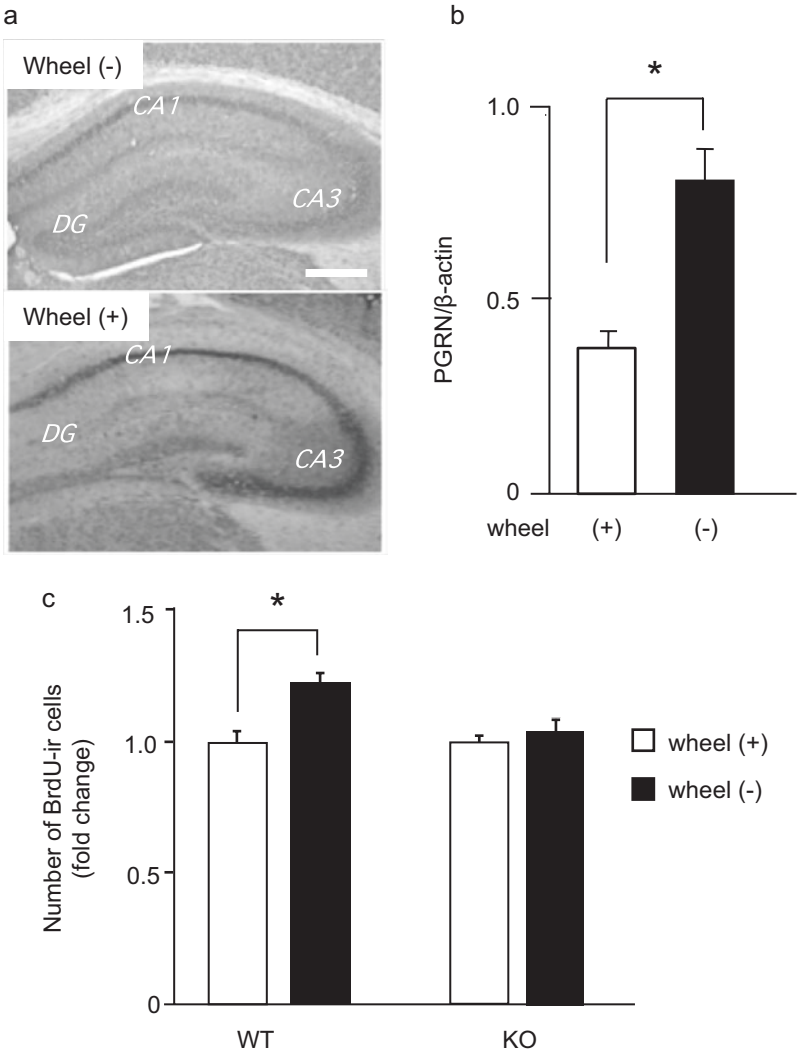

Fig. 3 Enhancive effect of PGRN on the hippocampal neurogenesis after voluntary running (**a**) Representative images of progranulin immunohistochemistry in the hippocampus of WT mice 4 weeks after being kept with or without a running wheel. Scale bar: 500 μm. (**b**) PGRN expression in the hippocampus and cerebellum of the mice 4 weeks after being kept with or without a running wheel, determined by western blotting. Each column and vertical bar represents the mean and SEM (n = 4). *P < 0.05, unpaired t-test. (**c**) Effect of voluntary exercise on the number of BrdU-ir cells in the SGZ of the hippocampus. Mice were kept individually with or without a running wheel in their cages for 6 weeks. Each column and vertical bar represents the mean and SEM (n = 5). *P < 0.05, analysis of variance followed by Tukey–Kramer's test

demonstrated that in the basal condition, the major source of PGRN is neurons and microglia. The increased PGRN-expression after wheel running was mainly observed in the NeuN-immunoreactive (ir) cells but not in the Ib-1-ir or GFAP-ir cells in the CA1, CA3, and hilus. Thus, the pyramidal neurons in CA1 and CA3 and interneurons in the hilus are possibly the major source of PGRN increased in the hippocampus responding to voluntary exercise. Previously, we have found that expression of hypothalamic PGRN is increased by estrogen in the brains of neonatal rats (Nedachi et al. 2011) and the hippocampus of mature rats (Chiba et al. 2007). Thus, these findings suggest that voluntary exercise, as well as estrogen, can induce the expression of PGRN in the brain.

As mentioned above, voluntary exercise has an enhancive effect on adult neurogenesis. Thus, to evaluate the possible involvement of PGRN in the enhancement of hippocampal adult neurogenesis by voluntary running, we put a running wheel into the home cage of WT and KO mice and kept them for 6 weeks. To label proliferating cells, bromodeoxyuridine (BrdU), a thymidine analogue was administrated 2 h before sampling. After 6-week voluntary running, the number of BrdU-ir cells in the SGZ of the WT mice was significantly increased in the exercised than the control mice (Fig. 3c). In the KO mice, however, voluntary exercise failed to increase the number of BrdU-ir cells. These findings imply that PGRN plays indispensable role in running-inducible enhancement of hippocampal neurogenesis, which is well consistent with our another finding that PGRN may stimulate hippocampal neurogenesis in rat brain (Chiba et al. 2007). One possible source of PGRN facilitating adult neurogenesis should be pyramidal cells and interneurons in the CA1 and CA3 regions and the hilus, respectively as those area showed strong running-inducible expressions of PGRN. Another possibility is that PGRN secreted by the neural progenitor cells in the SGZ contributes to the increase in neurogenesis, as we have found that neural progenitor cells derived from hippocampus produce considerable amounts of PGRN *in vitro* (Nedachi et al. 2011). Furthermore, PGRN has been reported to have a neurotrophic function on outgrowth of neurite (Van Damme et al. 2008). On the other hand, exercise increases the density of dendritic spine in CA1 pyramidal cells, which contributes to improvement of cognitive function (Stranahan et al. 2007).

Taken together, the upregulated PGRN in CA1 and CA3 and the hilus responding to voluntary exercise might be involved in maintenance and improvement of cognitive function resulting from promoting neurite development.

PGRN Plays a Protective Role in Hippocampal Neurogenesis against Immune Challenge by Suppressing Neuroinflammatory

Infectious stress and the subsequent increase of inflammatory cytokines suppress adult neurogenesis. We have previously demonstrated that PGRN suppresses excessive neuroinflammatory responses after traumatic brain injury (TBI) by decreasing

lysosomal biogenesis (Tanaka et al. 2013). Subsequently, we have evaluated the anti-inflammatory role of PGRN on the hippocampal neurogenesis (Ma et al. 2017) First, we evaluated hippocampal gene expression pattern of PGRN after intraperitoneal injection of bacterial endotoxin Lipopolysaccharide (LPS). LPS, a cell surface component of gram-negative bacteria, is broadly used for the experimental paradigms involving inflammatory and immune responses induced by infection. The expression level of PGRN was significantly increased 24 and 48 h after LPS injection. Since we have previously demonstrated that PGRN is mainly localized in lysosomes of activated microglia after TBI (Tanaka et al. 2013), we next performed double-immunostaining for PGRN with lysosome marker lysosomal-associated membrane protein 1 (LAMP1) or activated microglia marker CD68 in order to characterize the localization of PGRN in the hippocampus. Twenty-four hours after LPS injection, PGRN was well co-localized with CD68 in the DG. Moreover, immunoreactive areas for PGRN and LAMP were well correspondent. These results suggest that LPS-induced PGRN mainly localized in lysosomes of activated microglia.

Secondly, we assessed the function of PGRN in adult neurogenesis in the SGZ of the hippocampus under infectious condition. In addition to BrdU, we immunostained Ki67 as a marker of newborn cells. The numbers of immunoreactive cell for Ki67 and BrdU in the SGZ were significantly decreased both in WT and KO mice both 24 and 48 h after LPS injection, which is well corresponding with the previous studies reporting that adult neurogenesis is downregulated by various kinds of stressors. At 24 h after LPS injection, no significant difference was detected in the numbers of Ki67- or BrdU-ir cells in the SGZ between genotypes. However, the numbers of immunoreactive cells for both two markers were significantly smaller in KO mice than WT mice at 48 h after LPS injection (Fig. 4), which implies that PGRN-deficiency exacerbates the suppressive effect of infection stress on the hippocampal neurogenesis. Thus, PGRN is probably one of the potent factors protecting adult hippocampal neurogenesis against immune challenges.

We next evaluated the role of PGRN in the process of microglia activation. We performed immunostaining for Iba-1 and CD68 in the hippocampus of WT and KO mice 24 and 48 h after LPS treatment. As mentioned above, Iba-1 is a used as a general marker for microglia both in resting and activated conditions, while CD68 is a specific marker for the microglia in an activated condition. The Iba-1-ir area in the DG was significantly larger compared than saline-treated animals both 24 and 48 h after LPS injection in both WT and KO mice. However, there was no difference in the Iba-1-ir area between genotypes both in saline-treated and LPS-treated groups. On the other hand, the CD68-ir area of KO mice was significantly larger comparing with that of WT mice even in saline-treated animals. The mice of both genotypes showed significant increase of CD68-ir areas at both 24 and 48 h after LPS injection. In addition, the CD68-ir area in the DG of KO mice was significantly larger than that of WT mice. These findings suggest increased PGRN inhibit microglia from excessive activation.

Activated microglia are known to release various cytotoxic mediators such as inflammatory cytokines when they are stimulated by LPS administration (Laplante and Sabatini 2012; Settembre et al. 2012). Accordingly, we examined the gene

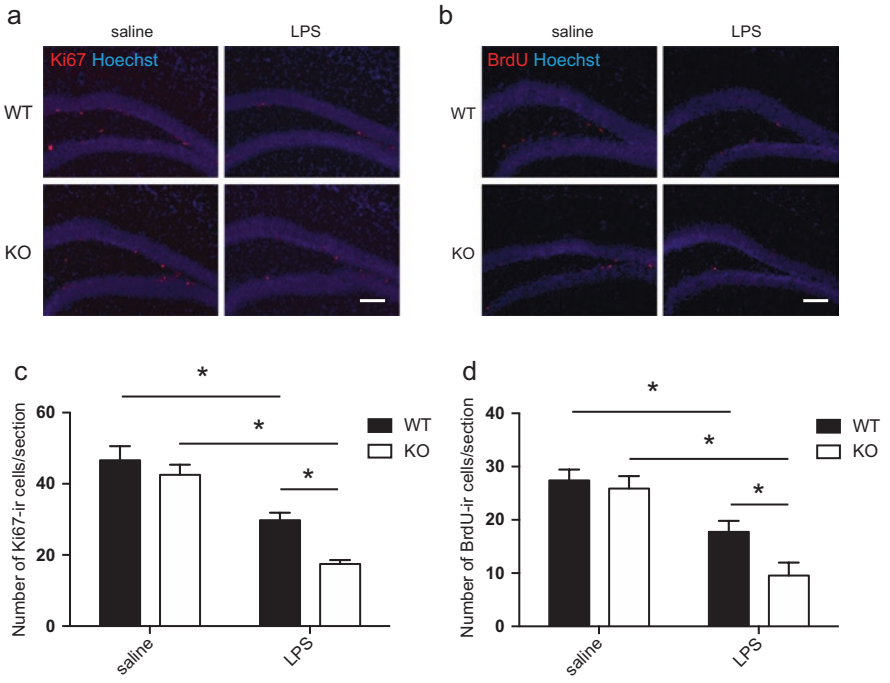

Fig. 4 Exacerbation of LPS-induced suppression of hippocampal neurogenesis in PGRN-deficient mice

(**a**, **b**) Representative images of immunohistochemistry for Ki67 (**a**) and BrdU (**b**) in the hippocampus of WT and PGRN KO mice 48 h after LPS administration. Nuclei were stained with Hoechst (blue). Scale bars, 100 μm. (**c**, **d**) Quantification of the number of Ki67 (**c**) and BrdU-positive cells (**d**) in the subgranular zone of the hippocampal dentate gyrus. Each vertical bar represents the mean and SEM (n = 5). *$p < 0.05$, Tukey's HSD test after two-way ANOVA

expression patterns of proinflammatory cytokines and microsomal prostaglandin E synthase-1 (mPGES-1) in the hippocampus to know the role of LPS-induced PGRN in regulation of expression of these genes. Regarding the genes of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), both of their expression levels were increased by LPS injection, although there was no significant difference between genotypes either in saline- or LPS-injected groups. The expression level of mPGES-1 was also increased by LPS in both genotypes. However, KO mice showed the higher mPGES-1 levels in LPS-injected condition. In addition, IL-6 expression level was increased by LPS only in KO mice. These data suggest that PGRN has a suppressive effect on the expression of IL-6 and mPGES-1 after LPS treatment.

Finally, we investigated whether there are differences in the expression levels of lysosomal genes in the hippocampus between WT and KO mice 48 h after LPS injection. We have focused on lysozyme M (Lyz2), macrophage expressed gene 1 (Mpeg1), cathepsin Z (Ctsz), and mammalian target of rapamycin (mTOR). In the saline-treated animals, the expression levels of Lyz2 and Mpeg1 were significantly

higher in KO than WT mice. LPS treatment significantly increased expression levels of those genes in both genotypes, and both genes were expressed significantly higher in KO mice than in WT mice. The LPS-induced increase of Ctsz expression level was observed only in KO mice, which was higher than that in WT mice. Although mTOR expression was significantly increased after LPS treatment in both genotypes, the increased level was much higher in KO mice. Previous studies have suggested that mTOR serves as a negative regulator of lysosomal biogenesis (Laplante and Sabatini 2012; Settembre et al. 2012). These results suggest that PGRN attenuates lysosomal biogenesis following LPS administration by enhancing activation of the mTOR signaling.

Hippocampal expression of PGRN is increased by infectious stress, which plays protective role in adult neurogenesis in the hippocampus against suppressive effect of infection. Besides, PGRN suppresses excessive neuroinflammatory responses related with overactivation of microglia after immune challenge, probably through decreasing lysosomal biogenesis involving mTOR activation.

Astrocyte-Specific Absence of Estrogen Receptor α in PGRN KO Mice

We have demonstrated that PGRN mediates some functions of estrogen including masculinization of the rodent brain and protection of hippocampal adult neurogenesis as written above. We also reported the strong induction of PGRN expression by estrogen in the rat brain (Chiba et al. 2007). Based on these findings, we have recently examined the localization of estrogen receptor α (ERα), a classic well-studied subtype of estrogen receptors, in the brain of KO mice by immunohistochemical analysis (Doke et al. 2016). A double-labeling immunofluorescence study for ERα and markers of each cell type was performed to characterize ERα-expressing cells in the brain of the mice sampled on the day of estrus and diestrus. ERα immunopositive cells were observed broadly in the brain, including the cortex, amygdala, hippocampus, thalamus, and hypothalamus of both WT and KO mice, regardless of the estrus stages. Most of NeuN-ir cells were also expressing ERα, whereas Iba1-ir was never co-expressed with ERα in both genotypes, which was well consistent with the previous studies. Interestingly, there was no co-localization of ERα with GFAP in five of six KO mice, while WT mice showed clear co-localization of these two markers (Fig. 5a). This result indicates that in astrocytes, PGRN plays a considerable role in ERα expression, regardless of the estrus stage. Accordingly, to investigate the possible involvement of endogenous estrogen in the regulation of ERα expression by PGRN in astrocytes, we performed double-labeling immunostaining of ERα and GFAP on the brains of immature female mice, adult ovariectomized female, and adult male. As consistent with cycling females, there was no co-localization of ERα and GFAP in the brains of KO mice of all three groups, while these markers co-localized well in all brains of WT mice (Fig. 5b), suggesting that estrogen is not involved in the regulation of ERα expression by PGRN.

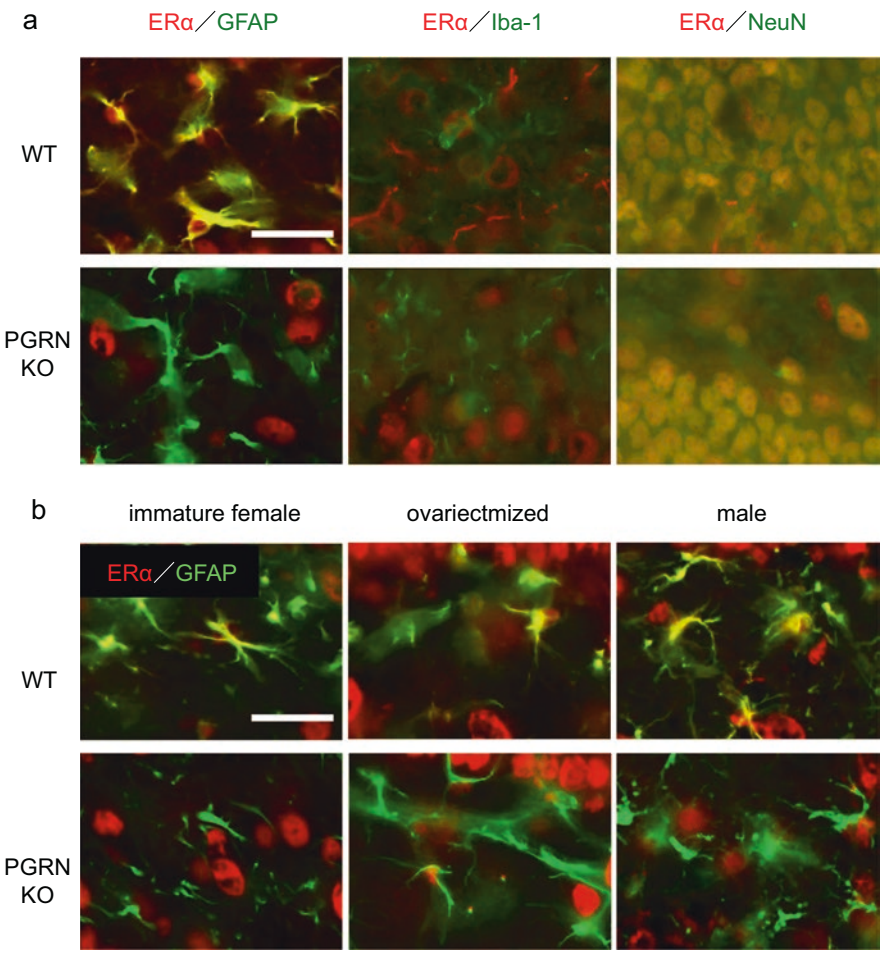

Fig. 5 Representative images of double immunostaining of ERα and cell markers in the hippocampus. (**a**) GFAP, Iba-1, and NeuN were detected as markers of astrocytes, microglia, and neurons, respectively. Co-localization of ERα and GFAP was observed only in WT mice. (**b**) None of immaturity, ovariectomy, or sex affected the lack of ERα in astrocytes of PGRN KO mice. Scale bars: 25 μm

As mentioned above, we have also reported that PGRN has anti-inflammatory and the resultant neuroprotective effects by demonstrating the enhancement of microglia activation and subsequent neuroinflammatory response induced by TBI (Tanaka et al. 2013, 2014). Since activated astrocytes are reported to activate microglia (Liu et al. 2011), the lack of ERα in astrocytes is possibly a major factor causing hyperactivation of microglia and neuroinflammation observed in brain of KO mice. These studies imply that PGRN controls ERα expression in astrocytes, which in turn mediates the anti-inflammatory function of estrogen in the brain. Moreover, estrogen is also known to stimulate astrocytes to release some kinds of

neuroprotective factors, such as glial cell-line derived neurotrophic factor (Platania et al. 2005; Xu et al. 2013), nerve growth factor (Xu et al. 2013), vascular endothelial growth factor (Barouk et al. 2011), and brain-derived neurotrophic factor (Xu et al. 2013). Interestingly, PGRN is expressed in neurons and microglia but not in astrocytes (Tanaka et al. 2013; Asakura et al. 2011). Astrocytes are known to interact with neurons through synaptic signal transduction (Benarroch 2005) and with microglia during neuroinflammation (Liu et al. 2011). Thus, PGRN possibly acts on neurons or microglia to regulate astrocytic ERα expression. Another possible mechanism is that PGRN secreted from neurons might affect astrocytes to regulate ERα expression, since PGRN is reported to work as a secretory glycoprotein (Van Damme et al. 2008).

In conclusion, PGRN produced in neurons and/or microglia works on astrocytes directly or indirectly to induce the expression of ERα, which probably mediates the function of estrogen to suppress neuroinflammation derived from brain damage.

Motor Dysfunction of the PGRN KO Mice

Our previous immunohistochemical study elucidated that PGRN is strongly expressed also in Purkinje cells in hippocampus (Matsuwaki et al. 2011). The well-established functions of cerebellum in motor coordination and motor learning lead us to evaluate the motor function of PGRN KO mice to investigate the possible role of PGRN in the motor function controlled by cerebellum. We performed rotarod tests on WT, HZ, and KO mice at the age of 3 months old (Matsuwaki et al. 2015). For each animal, three trials were performed on each of three successive days. Latency to fall from the rotating rod became longer with trials in all genotype. KO mice stayed on the rod significantly shorter than WT mice in the second and seventh trial (Fig. 6a). When we compared the mean duration on each day, there was a significant difference between WT and KO mice on day 3. These results indicate that PGRN deficiency impairs motor function, although without affecting the ability of kinesthetic learning. Petkau et al. have generated another line of PGRN KO mice and reported that their 8-month-old PGRN KO mice also showed impairments in some kinds of behavior tests including rotarod test (Petkau et al. 2012), corresponding with our present result.

PGRN is also expressed in peripheral motor neurons that connect to neuromuscular junctions (Ryan et al. 2009). In addition, PGRN is reported to enhance hypertrophy of myotubes of C2C12 mouse myogenic cells (Hu et al. 2012). Thus, there was a possibility that the significantly shorter duration of KO mice staying on rotating rod was attributed to growth defects in skeletal muscle caused by the PGRN deficiency. Thus, we performed morphometric analyses on tibialis anterior muscles. The result showed that the distributions of myofiber diameter were comparable between WT and KO mice, indicating that the growth of skeletal muscle was not defective in KO mice and there was no muscular weakness attributable to loss of muscle mass. In addition, as already mentioned above, we have previously demonstrated that loco-

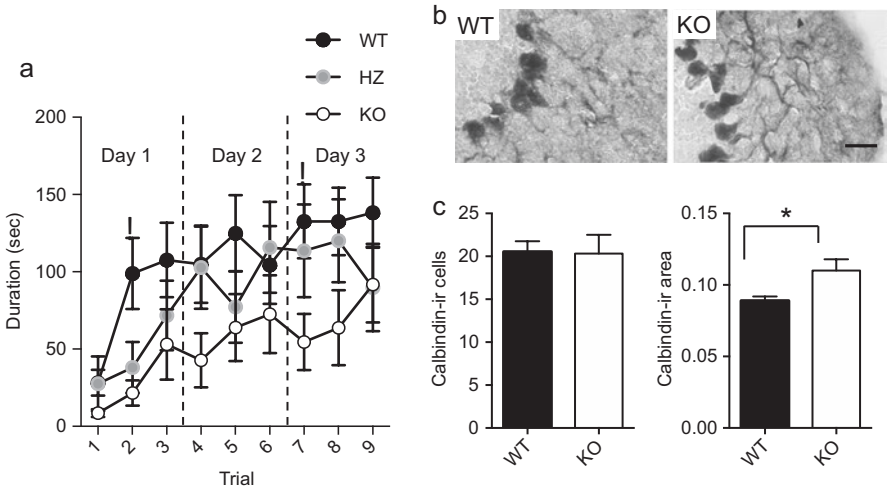

Fig. 6 Motor function and Purkinje cell morphology of PGRN-deficient mice
(**a**) Rotarod performance of WT, HZ, and KO mice. The rod was rotated at the speed of 30 rpm. For each animal, the duration on the rod was measured three times on each of three successive days. Each point and vertical bar represents the mean and SEM, respectively (n = 11 for each genotype). *P < 0.05 between WT and KO mice in the same trial ANOVA, followed by Tukey's multiple comparisons test. (**b**) Representative images of anti-calbindin immunohistochemistry in the cerebellums of WT and KO mice. Scale bar: 25 μm. (**c**) Summary of the numbers of calbindin-immunoreactive (ir) cells and the calbindin-ir area. The numbers of calbindin-ir cells in 600 μm of the Purkinje cell layer were counted in each of five slices and the mean values were calculated for each mouse. The calbindin-ir area was measured in five randomly selected areas (117 μm × 117 μm) in the molecular layer of five slices for each mouse and the mean value was calculated for each mouse. Each column and vertical bar represents the mean and SEM, respectively (WT, n = 6; KO, n = 5). *P < 0.05 (unpaired t-test)

motor activity of KO mice is comparable with that of WT mice in the open-field test (Kayasuga et al. 2007) or in measurement of voluntary running distance in a running wheel (Asakura et al. 2011). These results demonstrate that PGRN deficiency dose not impair growth of skeletal muscle or the resultant muscular weakness, and that the major deficit in motor function of KO mice is probably result from the impairment of motor coordination rather than decline of locomotor ability.

In 2006, there were two studies reporting that PGRN haploinsufficiency causes frontotemporal lobar degeneration (FTLD), the second most common dementia in human under the age of 65 (Baker et al. 2006; Cruts et al. 2006). However, gait abnormality is not the major clinical symptom of FTLD (Riedl et al. 2014), and a transgenic mouse model of FTLD with genomic fragments encoding human transactive response DNA-binding protein-43 (TDP-43), deposits of which can cause neurodegeneration in FTLD, does not present motor dysfunction until 32 weeks after birth (Swarup et al. 2011). However, two cases of neuronal ceroid lipofuscinosis (NCL), each involving homozygous mutations in GRN, have been reported recently (Smith et al. 2012). The major symptoms of NCL include visual loss, sei-

zures, bradykinesia, loss of cognitive function, and early death (Bennett and Rakheja 2013). In the results of our study, HZ mice did not show evident abnormality in motor function in the rotarod test, whereas homozygous KO mice have motor dysfunction. These results may suggest that heterozygous PGRN deficiency could cause FTLD-like symptoms, while homozygous PGRN deficiency may cause more severe NCL-like symptoms.

Higher Density of Dendrites of Purkinje Cells in the Cerebellum of PGRN KO Mice

Subsequently, we have analyzed the influence of PGRN deficiency by comparing the cerebellar histology of WT and KO mice. We performed Nissl staining and immunohistochemistry for calbindin as a marker for Purkinje cells. Immunostaining for calbindin, as well as Nissl staining, showed that KO mice do not have any evident difference in the layer structure of the Purkinje cells with WT mice. In addition, the number of calbindin-ir cells were comparable between genotypes. These results suggest that PGRN is not involved in the generation or the survival of Purkinje cells in the cerebellum. Surprisingly, however, the calbindin-ir area in the molecular layer of the cerebellum was significantly larger in KO mice than in WT mice (Fig. 6b, c), indicating the higher density of dendrites of Purkinje cells in KO mice. PGRN plays an considerable role in the maturation of the nervous system including outgrowth of neurite (Van Damme et al. 2008). Moreover, lack of PGRN decreases spine density and disrupts the synaptic connectivity of pyramidal neurons in the mouse hippocampus (Petkau et al. 2012). Thus, it was unexpected that PGRN KO mice showed the higher density of Purkinje cell dendrites. In the process of development of neuronal circuits, the number of synapses and the related neurites rapidly increase after birth and peaks before maturation. After this peak, pruning of the excessive synapses takes place to establish the matured neuronal circuits (Boothe et al. 1979). PGRN deficiency might impair this maturation process, which affects brain functions in adulthood such as anxiety tendency, cognitive functions, and motor functions.

The motor dysfunction and alteration in density of Purkinje cell dendrites suggest that PGRN is involved in the maturation of cerebellar neural networks, which in turn may affect motor function in mice. Heterozygous and homozygous PGRN KO mice might be of use as model animals for FTLD and NCL, respectively.

Conclusion

We have established a PGRN KO mouse line with depletion of all the exons but exon1. The KO mice are fertile and the number of weaned pups of them were comparable to that of WT mice. The male KO mice showed decreased ejaculation incidence, although they have normal latency and frequency of mount and intromission.

Moreover, anxiety and the size of LC, one of the anxiety-related nuclei, were increased in the male KO mice. These results imply that PGRN plays an important role during the maturation of male-specific neuronal circuits controlling male-type sexual behavior and anxiety. As PGRN expresses strongly in the hippocampus and the cerebellum, we have compared the histology and functions of those regions in the KO mice with those of WT. Accordingly, we have demonstrated that PGRN has a protective effect on the hippocampal neurogenesis by mediating the enhancive effect of voluntary exercise and by inhibiting the inflammatory stimuli. We have also demonstrated that PGRN is involved in development of Purkinje cells in the cerebellum, which may affect motor function in mice. PGRN is expressed in many regions in the brain and has a wide variety of neuroprotective effects not only during development but also in adulthood.

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Pleiotropic Protective Effects of Progranulin in the Treatment of Ischemic Stroke

Masato Kanazawa, Kunio Kawamura, Tetsuya Takahashi, and Takayoshi Shimohata

Abstract Stroke is one of the leading causes of morbidity and mortality in the world. The majority of strokes is ischemic. Unfortunately, there is still not well substantial treatment option to increase the survival and alleviate the outcome after ischemic stroke. In the central nervous system, progranulin (PGRN) is considered to play important roles in the maintenance of physiological functions. Mutations in the gene that encodes PGRN cause transactive response DNA-binding protein 43-positive frontotemporal lobar degeneration. Several studies have reported that PGRN exerts protective effects against ischemic brain injury. PGRN alleviates the impairments after acute focal cerebral ischemia by a variety of mechanisms, which we call "brain protection". This includes neuroprotection, suppression of neuroinflammation, and attenuation of blood-brain barrier disruption, i.e., vascular protection. PGRN has pleiotropic protective effects and is, therefore, an ideal candidate molecule for the therapy of stroke. We will accelerate the research towards further development of PGRN-based treatments of stroke.

Keywords Cerebral ischemia · Tissue plasminogen activator · Brain protection · Pleiotropic protective effects

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Introduction

Stroke is one of the leading causes of morbidity and mortality in the world. The majority (70–80%) of strokes is ischemic (Feigin et al. 2009). Tissue plasminogen activator (t-PA) is the only thrombolytic agent against acute ischemic stroke. The guidelines of the American Heart Association/American Stroke Association for the administration of t-PA were revised to extend the therapeutic time window to 4.5 h after the onset of symptoms in 2012. Recently, reperfusion therapies, such as intravenous thrombolysis and mechanical thrombectomy, are now established to alleviate functional outcome (Goyal et al. 2016). However, the percentage of patients being eligible for these treatment options is still only 5% of all patients with acute ischemic stroke, because of the very narrow therapeutic time window (Writing Group Members et al. 2016). A pooled analysis demonstrated that the risks of fatal and serious symptomatic hemorrhagic transformation (HT) increases with later initiation of the treatment (Lees et al. 2010). Therefore, a reduction of the HT incidence after t-PA treatment is an important therapeutic strategy after ischemic stroke, which will further extend the therapeutic time window, increase the number of patients who are eligible for t-PA treatment, and raise the probability of achieving excellent outcomes.

"Single-target" therapies may be insufficient because ischemic cerebral injury involves several mechanisms. It has been proposed that therapeutic strategies should target multiple cell types to enhance protection and recovery (Moskowitz et al. 2010). We suggest that brain protection, which includes vascular protection, neuroprotection, and anti-inflammation, is an ideal therapeutic strategy for ischemic stroke (Fig. 1). We, among other groups, have reported that progranulin (PGRN) exerts pleiotropic protective effects against ischemic brain injury (Tao et al. 2012; Egashira et al. 2013; Jackman et al. 2013; Kanazawa et al. 2015). PGRN might be a novel therapeutic target that provides brain protection.

In this review, we describe that PGRN exert its protective effects in combination with t-PA treatment in cerebral ischemia. In addition, we briefly outline the brain protective strategies by which PGRN attenuates HT after t-PA treatment and improves the therapeutic outcomes after cerebral ischemia.

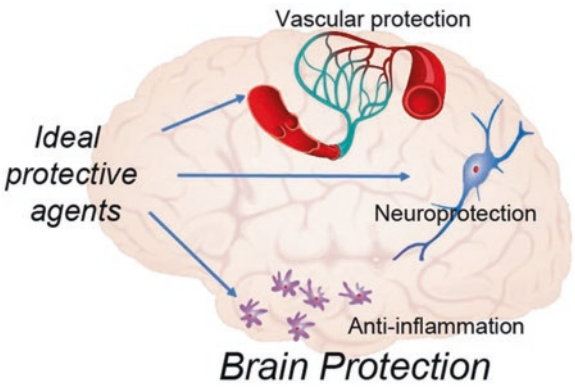

Fig. 1 Schema of ideal protective agents after cerebral ischemia

Pleiotropic Protective Effects of PGRN

PGRN is a secreted N-linked glycoprotein growth factor upregulated by estrogen and implicated in sex difference (Palfree et al. 2015; Suzuki et al. 2009), tissue regeneration, wound repair, and inflammation (He et al. 2003; Chitramuthu et al. 2017; Kao et al. 2017). PGRN contains seven and a half repeats of cysteine-rich granulin motifs (granulin A to G) separated by linker regions (reviewed in Chitramuthu et al. 2017; Kao et al. 2017).

In the central nervous system, PGRN is considered to play important roles in the maintenance of physiological functions and in neuronal survival for the following reasons: first, primary neurons derived from PGRN knock-out (KO) mice show reduced survival (Kleinberger et al. 2010); second, PGRN has been shown to exhibit neurotrophic activity *in vitro* (Van Damme et al. 2008) and *in vivo* (Laird et al. 2010), especially granulin C and granulin E peptides promote neuronal survival and neurite outgrowth in neocortical, hippocampal, and motor neurons *in vitro* (Gao et al. 2010; Gass et al. 2012); third, mutations in the gene that encodes PGRN cause transactive response DNA-binding protein 43 (TDP-43)-positive frontotemporal lobar degeneration (FTLD)-TDP, a neurodegenerative disorder with an autosomal dominant inheritance pattern (Baker et al. 2006; Cruts et al. 2006).

PGRN has been shown to be involved in anti-inflammation after ischemic brain injury. Previous studies demonstrate that PGRN is induced in activated microglia after spinal cord injury (Naphade et al. 2010) and traumatic brain injury (Tanaka et al. 2013), suggesting that the induction of PGRN reflects the microglial response. In ischemic brain injury, microglia have been shown to extend the infarct via inflammation in the acute phase (Mabuchi et al. 2000; Yenari et al. 2010), while a subpopulation of microglia called M2-like microglia contribute to endogenous anti-inflammatory protection (reviewed in Ma et al. 2017; Kanazawa et al. 2017a) M2-like microglia might secrete PGRN and suppress inflammation (Kanazawa et al. 2015, 2017a; Ma et al. 2017).

PGRN might play a role in vascular protection after focal cerebral ischemia via suppression of blood-brain barrier (BBB) disruption. Studies have demonstrated that intraventricular and intravenous administration of recombinant PGRN (rPGRN) might suppress cerebral edema in a mouse and a rat model of transient focal cerebral ischemia (Egashira et al. 2013; Kanazawa et al. 2015), and that PGRN KO mice may be prone to post-ischemic BBB disruption (Jackman et al. 2013; Kanazawa et al. 2015). Therefore, PGRN plays an important role in BBB maintenance.

The Mechanisms of Pleiotropic Protective Effects of PGRN in Cerebral Ischemia

PGRN is an ideal therapeutic molecule for brain protection especially after an ischemic stroke. It can exert a positive therapeutic effect in acute focal cerebral ischemia via neuroprotection, suppression of inflammation, and vascular protection (i.e., the regulation of BBB functions).

Neuroprotection After Cerebral Ischemia

PGRN protects neurons after acute focal cerebral ischemia based on analyses using PGRN- overexpressing transgenic mice (Tao et al. 2012) and PGRN KO mice (Jackman et al. 2013; Kanazawa et al. 2015). We have previously demonstrated that ischemic neuronal injury might be caused in part by cleavage and cytoplasmic redistribution of TDP-43, a key protein in FTLD-TDP and amyotrophic lateral sclerosis (Kanazawa et al. 2011a). PGRN can suppress the cleavage of TDP-43 via inhibition of caspase-3 (Kanazawa et al. 2011a; Zhang et al. 2007). This raises the possibility that PGRN may prevent ischemic neuronal injury via preservation of TDP-43 functions. We have shown that exogenous rPGRN could suppress neuronal cell death under conditions of oxygen-glucose deprivation (Kanazawa et al. 2015). The neurotrophic effects of PGRN might be explained in part by the inhibition of abnormal cytoplasmic redistribution of nuclear TDP-43 (Kanazawa et al. 2011a; Zhang et al. 2009). Stated another way, a decreased level of full-length PGRN might cause loss of function of TDP-43 in neurons, resulting in neuronal damage. In addition, PGRN showed apoptotic inhibition by activating PI3k/Akt pathway in subarachnoid hemorrhage (SAH) model in rats (Li et al. 2015). Knockdown of the PGRN receptor sortilin abolished Akt activation, increased Bcl-2 levels, and reduced cleaved caspase-3 levels, and prevented the functional recovery induced by rPGRN treatment after SAH. PGRN might mediate neuroprotection via inhibition of caspase-mediated apoptosis (Fig. 2).

Anti-inflammatory Effects After Cerebral Ischemia

PGRN reduces inflammation after acute focal cerebral ischemia. We have shown that PGRN may upregulate the anti-inflammatory cytokine interleukin (IL)-10 (Kanazawa et al. 2015). Previous studies report that the level of IL-10 in glial cells from

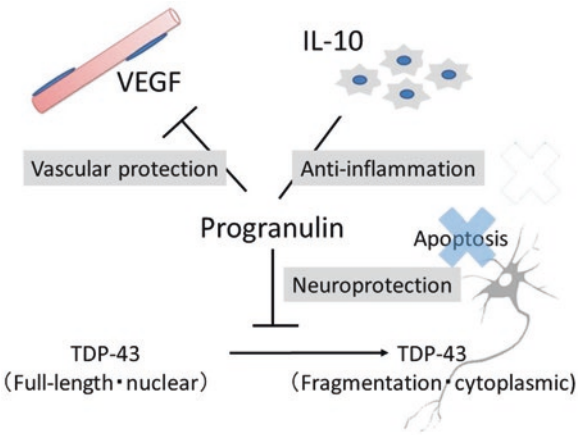

Fig. 2 Therapeutic mechanisms of progranulin in cerebral ischemia *IL* interleukin, *TDP-43* transactive response DNA-binding protein 43, *VEGF* vascular endothelial growth factor

PGRN-overexpressing transgenic mice is higher than that from wild-type (WT) mice (Tao et al. 2012), and that macrophages/microglia from PGRN KO mice release less IL-10 than those from WT mice when exposed to bacterial lipopolysaccharide (Yin et al. 2010) and oxygen-glucose deprivation (Kanazawa et al. 2015). Very recently, it has been reported that PGRN has protective effects in focal cerebral ischemia via suppression of nuclear factor-kappa B (NF-κB) signaling and subsequent downregulation of pro-inflammatory cytokines (Shu et al. 2018). Another group demonstrated that mRNA levels of tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, and transforming growth factor beta (TGF-β) after ischemic brain injury are not correlated with the PGRN KO condition (Jackman et al. 2013). Unfortunately, this study did not investigate IL-10 levels. In addition, a previous study suggests that PGRN might suppress recruitment of neutrophils associated with focal cerebral ischemia (Egashira et al. 2013). SAH patients as well as rats in an SAH model showed significantly decreased levels of PGRN in the cerebrospinal fluid and the brain, respectively (Zhou et al. 2015a). These studies also showed that the levels of pro-inflammatory cytokines were markedly elevated. PGRN alleviates brain injury after SAH possibly via inhibition of neutrophil recruitment. However, other groups have shown that PGRN is not involved in neuroinflammation (Jackman et al. 2013) and recruitment of neutrophils related to acute focal cerebral ischemia (Kanazawa et al. 2015). Under conditions of traumatic brain injury, PGRN KO and WT mice exhibit no significant difference in the number of microglia/macrophages (Menzel et al. 2017). However, PGRN KO mice show increased transcription of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, and decreased transcription of the anti-inflammatory cytokine IL-10. The role of PGRN in neuroinflammation after ischemic brain injury is controversial and might depend on the model used. Probably, PGRN induces a pro- or anti-inflammatory phenotype via effects at the molecular level (Fig. 2).

Furthermore, administration of rPGRN significantly alleviates the inflammatory responses caused by renal ischemia/reperfusion injury in mice (Zhou et al. 2015b). PGRN also alleviates inflammatory responses in various disease models such as inflammatory bowel disase (Wei et al. 2014) and asthma (Chiba et al. 2018). In addition, Atsttrin, an engineered protein composed of three PGRN fragments, exhibits selective TNF receptor binding (Tang et al. 2011; Liu 2011). PGRN and Atsttrin prevent inflammation in mouse models of arthritis and inhibit intracellular signaling activated by TNF-α. Therefore, it can be concluded that PGRN suppresses inflammation in multiple organs.

Vascular Protection

PGRN can attenuate BBB disruption after acute focal cerebral ischemia. A previous report has shown that PGRN is induced in the capillary endothelium of wound granulation tissue and promotes the mitosis and migration of adult dermal microvascular cells (He et al. 2003), although PGRN is not expressed in the healthy endothelium (Daniel et al. 2000). We observed endothelial expression of PGRN (Kanazawa et al.

2015) and hypothesized that the expression of PGRN in endothelial cells may be involved in vascular protection or repair after ischemic injury. Indeed, cerebral edema volume after focal cerebral ischemia is larger in PGRN KO mice than in WT mice (Kanazawa et al. 2015). BBB disruption was observed after cerebral ischemia more severe in PGRN KO mice than in WT mice (Jackman et al. 2013). Regarding the mechanism by which PGRN regulates vascular permeability, we considered based on the findings from immunostainings using WT and PGRN KO mice that cerebral edema is not caused by recruitment of neutrophils and microglia (Kanazawa et al. 2015). Another group demonstrated the involvement of the platelet-derived growth factor (PDGF) receptor pathway using PGRN KO mice (Jackman et al. 2013). We proposed as another possibility that PGRN might regulate vascular permeability via vascular endothelial growth factor (VEGF) pathway because we found a more prominent expression of VEGF in PGRN KO mice than in WT mice after focal cerebral ischemia. In addition, PGRN KO microglia itself secretes VEGF and the conditioned media from PGRN KO microglia synergistically stimulates to secrete VEGF from PGRN KO astrocytes after OGD. PGRN might inhibit cerebral edema via nuclear factor-kappa B (NF-κB) (Egashira et al. 2013), which activates VEGF transcription (Yoshida et al. 1998). PGRN may regulate vascular permeability by inhibiting microglial production of molecules that activate the NF-κB-VEGF signaling pathway (Fig. 2).

Elevated levels of PGRN have a significant biological effect on vessel growth that might be independent of VEGF (Tao et al. 2012). Some of the effects of a PGRN KO in the penumbral blood vessels might be directly mediated by PGRN loss rather than via increased VEGF expression (Jackman et al. 2013). Furthermore, administration of rPGRN results in a remarkable suppression of the lipopolysaccharide-induced induced permeability increase in pulmonary capillaries (Guo et al. 2012; Yu et al. 2016). PGRN can effectively ameliorate the edema after brain and lung injury, suggesting a potential for PGRN-based therapy to treat clinically relevant diseases in multiple organs.

Expression Changes of PGRN After Cerebral Ischemia

In the healthy cerebral cortex, PGRN can be detected within the neuronal cytoplasm in a punctate pattern (Fig. 3), (Kanazawa et al. 2015; Kao et al. 2017) and double immunostaining reveals that PGRN is colocalized with marker proteins for the endoplasmic reticulum, Golgi apparatus, and lysosome. Under these conditions, microglia or blood vessels do not express PGRN.

We demonstrated *in vivo* the dynamic changes in PGRN in neurons, microglia, and endothelial cells after ischemia, including decreased levels of PGRN expression in neurons within the ischemic core, increased levels of PGRN expression in surviving neurons, as well as induction of PGRN expression in microglia and endothelial cells in the ischemic penumbra (Figure 3) (Kanazawa et al. 2015). Importantly, the number of PGRN-positive microglia was increased at 24 h and markedly increased

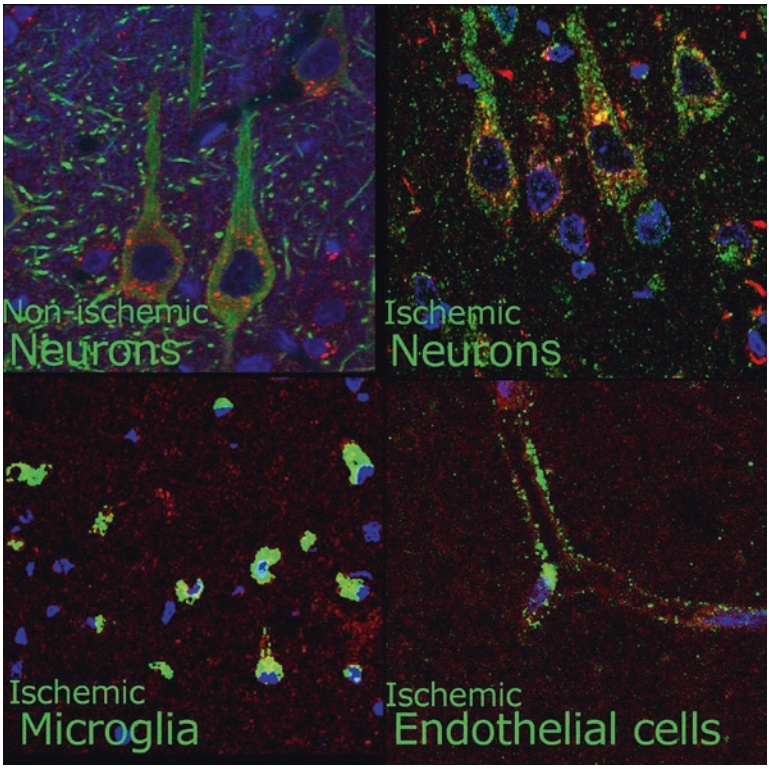

Fig. 3 Changes of progranulin expression after cerebral ischemia
Triple labeling in non-ischemic and ischemic regions of the cerebral cortex visualized by confocal microscopy. Cells were stained for microtubule-associated protein 2 (neurons), CD68/ED1 (microglia), or von Willebrand factor (endothelial cells, all shown in green) in addition to PGRN (red) and 4′,6′-diamidino-2-phenylindole (DAPI; blue)

at 72 h after reperfusion, especially on the border between the ischemic core and penumbra close to the ischemic core.

Therapeutic Benefits of PGRN After Cerebral Ischemia

Therapeutic benefits of PGRN after ischemic stroke have been reported (Tao et al. 2012; Egashira et al. 2013; Jackman et al. 2013; Kanazawa et al. 2015). In an editorial comment of the journal *Brain*, PGRN-based treatments were proposed (Zhao and Bateman 2015).

To examine the therapeutic benefits of PGRN after ischemic brain injury, we used a rat autologous thromboembolic model (Okubo et al. 2007), which develops cerebral edema and hemorrhagic transformation when t-PA is administered beyond the therapeutic time window (Kanazawa et al. 2015; 2011b; Kawamura et al. 2014).

We demonstrated for the first time that intravenous administration of rPGRN shows therapeutic effects regarding the volumes of the cerebral infarct and the consecutive edema, the HT, and the prognosis (Kanazawa et al. 2015). Again, given the therapeutic efficiency of rPGRN after ischemic stroke (Kanazawa et al. 2015), rat models of SAH (Li et al. 2015), and experimental traumatic brain injury (Zhou et al. 2015a), PGRN is due to its pleiotropic protective effects an ideal candidate molecule to treat brain injuries.

The Future Direction of PGRN-Based Therapies After Ischemic Stroke

The elevated serum PGRN levels are a negative predictor for the outcome of stroke patients (Xie et al. 2016). To the contrary, the levels of PGRN in cerebrospinal fluids decreased in the patients with SAH (Shu et al. 2018). The clinical significance of the PGRN levels are unknown. However, at least, PGRN could play an important role in the therapy of stroke patients.

Therapeutic approaches using PGRN are ideal because of its pleiotropic protective mechanisms. M2-like microglia secreted PGRN might suppress inflammation, and promote tissue recovery (Kanazawa et al. 2015). We have recently shown that transplantation of primary M2-like microglia preconditioned by 18 h oxygen-glucose deprivation prompt improving functional outcome after cerebral ischemia (Kanazawa et al. 2017b). This result is the possibility that cell source of PGRN might be a candidate for cell therapies against ischemic stroke. Very recently, the neuroprotective effects of PGRN after cerebral ischemia was reported by reducing endoplasmic reticulum stress and NF-κB activation in reactive astrocytes (Shu et al. 2018). The complicated therapeutic mechanisms of PGRN still need to be fully elucidated. Moreover, clinical trials which will evaluate the effects of PGRN-based treatments are needed. We will accelerate research towards further development of PGRN-based treatments of stroke.

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New Therapeutic Approaches Against Ocular Diseases

Yoshiki Kuse, Shinsuke Nakamura, and Hideaki Hara

Abstract Ophthalmology aims to enable patients with visual disorders to improve their visual acuity through cutting-edge medicine including retinal prosthesis using engineering methods and gene therapy. It is also engaged in a clinical trial using induced pluripotent stem (iPS) cells; however, there is still a long way to go before retinal regenerative medicine shows sufficient clinical results to improve visual function. Therefore, it is important that neuroprotective therapeutics, which could prevent disease progression, or regenerative medicine to recover retinal function, are ready for practical use in the future. In recent years, it has been reported that progranulin-activated cell signaling pathways regulate excitotoxicity, oxidative stress, and synaptogenesis, and are now gaining attention as a new target gene in the central nervous system (CNS). Despite being a peripheral organ, the retina or neural portion of the eye is actually part of the CNS. Here, we summarize the current understanding of the involvement of progranulin in retinal cells. In addition, many roles of progranulin on the survival and development on retinal cells have been recently revealed, which may lead to new approaches in both retinal neuroprotection and regeneration.

Keywords Adipose-derived stem cells · Inflammation · Neurogenesis · Photoreceptor cell · Progranulin · Retinal degeneration · Visual acuity

Introduction

Visual impairment occurs in elderly people. The number of people with visual disorders is increasing with our ageing society. An estimated 253 million people live with vision impairment; 36 million are blind and 217 million have moderate to severe vision impairment. Moreover, 81% of people who are blind or have visional impairment are aged 50 years and above (Bourne et al. 2017). The retina is

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important in vision to receive and recognize color and brightness. Rod and cone photoreceptors are specialized neurons that receive light. The process of vision is initiated by converting patterns of light energy into graded electrical potentials by photoreceptors. Retinal photoreceptor cell loss can severely impair vision. Age-related macular degeneration (AMD) and retinal pigmentosa (RP) are associated with photoreceptor degeneration, and there are currently no effective drug therapies. The retinal pigment epithelium (RPE) is located near photoreceptors and supports them. The pathological processes in eyes with non-exudative age-related macular degeneration (AMD) induce RPE atrophy (Liang and Godley 2003) and enhance photoreceptor cell degeneration caused by excessive light exposure and oxidative stress (Shahinfar et al. 1991; Beatty et al. 2000).

Recently, induced pluripotent stem (iPS) cell-derived RPE sheets were transplanted into an AMD patient autograft (Mandai et al. 2017). Several groups in human clinical trials have shown that RPE allografts cannot survive because of immune rejection. It is observed that loss of visual function and development of an exudative response and so on. To achieve this, human iPS-RPE cells using human leukocyte antigen (HLA) homozygote allografts are now being examined (Sugita et al. 2016a, b). However, transplantation did not improve the photoreceptor function, which was already decreased with the progression of ocular disease. Other reports show the transplantation of immature retinal cells into host retina because the mature retinal cells could not integrate into the host retina (MacLaren et al. 2006). In particular, the integration of transplanted cells to host retina is difficult although transplantation has succeeded (Gust and Reh 2011). New approaches are required for the protection or regeneration of retinal neurons such as photoreceptors. Several candidates have been found in many laboratory investigations (Williams et al. 2014; Gehrs et al. 2010; Bavik et al. 2015).

Protective Effect of Progranulin Against Photoreceptor Cell Degeneration

Progranulin Derived from Adipose-Derived Stem Cells

Mesenchymal stem cells (MSCs) have the multipotency and self-renewing capacity. MSCs were first found in bone marrow. Recently, a new source of MSCs was isolated from adipose tissue (Zuk et al. 2001). Adipose-derived stem cells (ASC) can be collected in large quantities with low invasiveness. ASCs can also be stably isolated and have shown a high proliferative capacity (Kern et al. 2006; Schubert et al. 2011). In clinical practices, ASCs are used for breast reconstruction and the safety has been confirmed (O'Halloran et al. 2017).

However, it is poorly understood whether ASC therapy is valid for retinal diseases. Excessive light exposure causes retinal photoreceptor degeneration and is used for the pathological model of AMD (Cachafeiro et al. 2013; Rattner et al. 2008; Gu et al. 2007). ASCs showed a protective effect against light-induced retinal degeneration (Tsuruma et al. 2014). ASCs improve retinal function and outer nuclear layer thickness decreased by light exposure. Therefore, ASCs preserve the photoreceptor cells in the model of AMD. Other reports show that human ASCs also protect against photoreceptor degeneration (Sugitani et al. 2013). MSCs derived from bone marrow are benefit to retinal damage (Labrador-Velandia et al. 2016).

It has been reported that mouse ASCs engraftment occurred in the retina using enhanced green fluorescent protein (EGFP) transgenic mouse-derived ASCs. Engraftment did not occur because GFP was not observed in the retina after intravitreal injection of ASCs (Tsuruma et al. 2014). Therefore, ASCs secreted factors could exert a protective effect against photoreceptor cell degeneration. ASC-conditioned medium (ASC-CM) also suppresses photoreceptor cell death induced by light exposure *in vivo* and *in vitro*. However, mature adipose medium (MA)-CM does not show a protective effect against photoreceptor cell damage. ASC secreted factors are analyzed by cytokine array to reveal the contribution of each factor to photoreceptor cell protection.

Progranulin was identified as a rich secreted protein in our study using ASCs (Tsuruma et al. 2014). Progranulin can provide a protective effect on neuronal cells through Wnt signaling (Rosen et al. 2011). ASC-CM contains a high concentration of progranulin (75 fold ASC-CM equals progranulin 574.15 ng/mL) compared to MA-CM. An equal amount of progranulin to 75 fold ASC-CM suppressed photoreceptor cell death induced by light exposure *in vivo* and *in vitro* (Tsuruma et al. 2014).

Protective Mechanism of Progranulin

Progranulin is reported to inhibit the tumor necrosis factor (TNF) receptor (Tang et al. 2011). It is controversial whether progranulin directly interacts with the TNF receptor (Chen et al. 2013; Cerezo et al. 2015). Regardless, progranulin inhibits the TNF receptor signaling and is a possible new therapeutic target for rheumatoid arthritis and etanercept (Cerezo et al. 2015; Liu 2011). Although progranulin exerts a protective effect against photoreceptor degeneration through the suppression of the TNF receptor, the neutralizing antibody for TNF receptor does not have a therapeutic effect on light-induced photoreceptor damage *in vitro* (Tsuruma et al. 2014), suggesting that another signaling pathway is associated with the effect of progranulin. The signaling was investigated using a phosphor-receptor tyrosine kinase array kit, and progranulin activates the HGF receptor within 5 min after its addition to

cells (Tsuruma et al. 2014). Progranulin may directly interact with HGF receptors. Another report shows that the expression of hepatic HGF receptor is modulated by progranulin (Li et al. 2010). The knockdown of GrnA in zebrafish, an orthologue of mammalian progranulin, downregulates the expression of the HGF receptor. Taken together, progranulin exerts a protective effect against photoreceptor cell damage through HGF receptors.

The downstream signaling is partly identified and is associated with the protective effect of progranulin. Progranulin increases the phosphorylation of cAMP response element binding protein (CREB) and a downstream target of extracellular regulated kinase (ERK) (Tsuruma et al. 2014). CREB is phosphorylated prior to the phosphorylation of ERK. ERK, protein kinase A, and PKC regulate the expression of CREB. The specific inhibitor (U0126, H-89, and Go6976, respectively) of each signaling is used to identify the signaling induced by progranulin. Go6976 only inhibited the protective effect of progranulin. Therefore, progranulin contributes to photoreceptor cell protection through the PKC-CREB pathway and possibly HGF receptor signaling (Fig. 1).

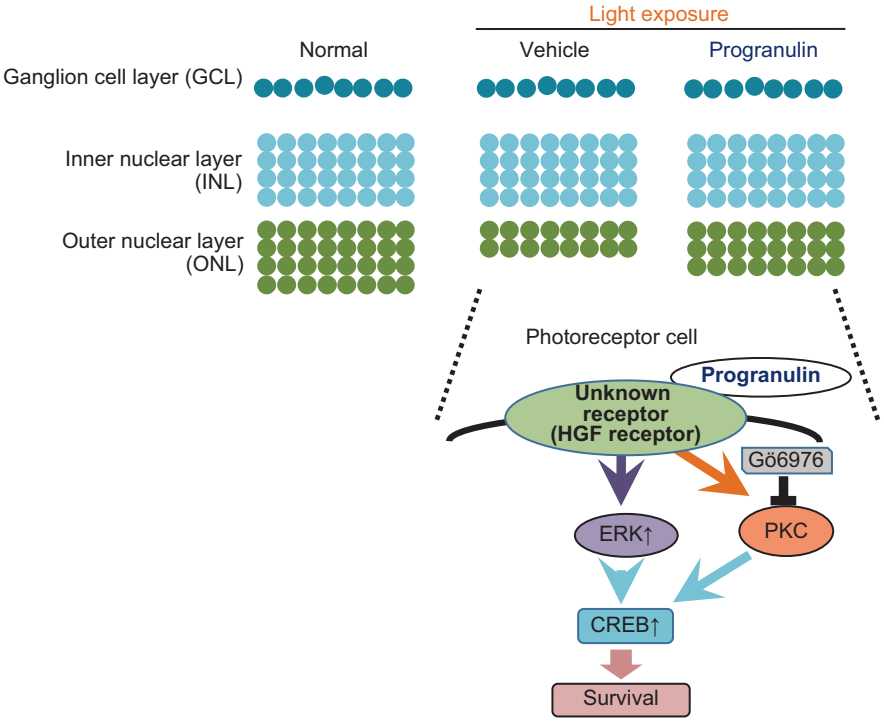

Fig. 1 The protective effect and mechanism of progranulin against retinal photoreceptor cell damage. Progranulin-enhanced phosphorylation of the HGF receptor and may activate CREB via the PKC pathways. As a result, CREB activation leads to photoreceptor cell survival

Regenerative Effect of Progranulin

Potential of Regenerative Medicine Against Retinal Degeneration

As mentioned above, a new approach is required for the treatment of visual impairment. The stem cell niche is focused on therapy for multiple diseases (Labrador-Velandia et al. 2016; Lane et al. 2014). Adipose tissue is one stem cell niche observed in MSC. In the central nervous system (CNS), neuronal cells are constitutively generated, which is called neurogenesis (Arvidsson et al. 2002; Eriksson et al. 1998). However, it has long been thought that mammalian neurons are unable to regenerate after being damaged. It was recently reported that limited neuronal regeneration can occur in the CNS, such as the brain and retina in adult mammals (Harada et al. 2011; Osakada et al. 2007; Ooto et al. 2004). In the retina, Müller glia have the potential as retinal stem cells to dedifferentiate and proliferate to retinal precursor cells after injury (Osakada et al. 2007; Ooto et al. 2004). Subsequently, some of these retinal precursor cells migrate to other any retinal cell layers and differentiate into retinal cells (Ooto et al. 2004; Fausett and Goldman 2006). In zebrafish, retinal regeneration occurs completely after retinal injury (Fausett and Goldman 2006). Some of the key regulators were identified in the regeneration after zebrafish retinal injury (Goldman 2014). However, it remains unknown how mammalian retinal regeneration occurs after retinal injury.

The retinal protection is provided by the ASC treatment after retinal damage. It is possible that ASC-secreted factors can accelerate retinal regeneration as well as retinal protection. The potential regenerative effect of ASC-CM is assessed in a retinal damage model using N-Methyl-N-nitrosourea (MNU), which causes photoreceptor cell specific damage. After MNU treatment, ASC-CM is treated using a frequent intravitreal injection. ASC-CM increased the newly generated BrdU positive cells in the outer nuclear layer (ONL), which increases photoreceptor cells after retinal damage compared to the vehicle-treated group (Kuse et al. 2016). These BrdU positive cells are rhodopsin (photoreceptor marker) positive. Therefore, ASC-CM increased newly generated photoreceptor cells after retinal damage. As mentioned above, ASC-CM contains a high concentration of progranulin (75 fold ASC-CM equals progranulin 574.15 ng/mL) (Tsuruma et al. 2014). Whether progranulin mimics the effect of ASC-CM was examined in a similar protocol. Progranulin is treated after light-induced retinal damage, a better model reflected to a pathology of AMD than of MNU. Progranulin increases the number of BrdU positive newly generated cells in the ONL (approximately fourfold increase) compared to the vehicle-treated group (Kuse et al. 2016). However, BrdU positive cells are not rhodopsin positive, although these cells are present in the ONL. In other words, proliferative cells exist in the ONL but they do not differentiate into photoreceptor cells. BrdU positive cells are co-stained with various cell markers such as retinal precursor cells and glial cells to identify which cells proliferate. BrdU positive cells in the ONL are not co-localized with glial fibrillary acidic protein (GFAP), a marker

specific for astrocytes and ionized calcium binding adaptor molecule 1 (Iba-1), a marker specific to microglia. Therefore, it is suggested that progranulin does not induce gliosis after retinal damage. Rx (retinal homeobox protein) is selected as a retinal precursor cell marker. Rx is associated with retinal development and is expressed in retinal precursor cells (Marquardt and Gruss 2002; Furukawa et al. 1997a). Some Rx and BrdU positive cells are observed in the ONL in the progranulin treated group but not in the vehicle-treated group (Kuse et al. 2016). Moreover, Rx mRNA is co-localized with Rx protein and BrdU positive cells in the ONL. This result suggested that the increase in BrdU positive cells in the ONL resulting from progranulin treatment are partly Rx positive retinal precursor cells. Other retinal precursor cell markers were investigated. Nestin is a marker of neural precursor cells. It is reported that nestin is expressed when an injury induces Müller glial neural stem cell-like properties (Osakada et al. 2007). Nestin expression is not altered between the vehicle and progranulin-treated groups. Sox2 (sex determining region Y-box 2) is a stem cell marker. A few BrdU and Sox2 double-positive cells were observed in the progranulin-treated group, but none of these cells were observed in the vehicle-treated group (Kuse et al. 2016). Moreover, cone-rod homeobox protein (CRX) indicates retinal photoreceptor precursor cells (Furukawa et al. 1997b). CRX expression is observed in the progranulin-treated group but not in the vehicle-treated group (Kuse et al. 2016). These results suggest that progranulin increases the newly-generated retinal precursor cells in the ONL after retinal damage but does not differentiate to photoreceptor cells (Fig. 2). The combination of Wnt and retinoic acid or valproic acid promotes differentiation to photoreceptor cells (Osakada et al. 2007). Valproic acid treatment after overexpression of Sox2 promotes the maturation of neurons in the brain (Niu et al. 2013). Retinal regeneration proceeds dedifferentiation (of Müller glia), proliferation (of retinal precursor cells), and differentiation. The combination therapy with progranulin could promote the regeneration of photoreceptor cells after injury.

Photoreceptor Cell Regenerative Effect of Progranulin

The regenerative effect of progranulin is likely associated with Wnt signaling and the HGF receptor. Progranulin can modulate the Wnt signaling pathway, which is associated with cell proliferation and development (Rosen et al. 2011; Korade and Mirnics 2011; Inoue et al. 2006). Wnt3a increases neurogenesis in the hippocampus and also promotes retinal regeneration in rats and mice (Osakada et al. 2007; Lie et al. 2005). Another report shows that progranulin is associated with muscle regeneration increasing the pool of myogenic progenitor cells through the HGF receptor (Li et al. 2013).

Retinal development is initiated at the embryonic stage and progresses to the postnatal stage. Development is essential for normal eye formation. The process of retinal development (such as precursor cell proliferation and differentiation to mature cells) is similar to retinal regeneration. The involvement of progranulin in

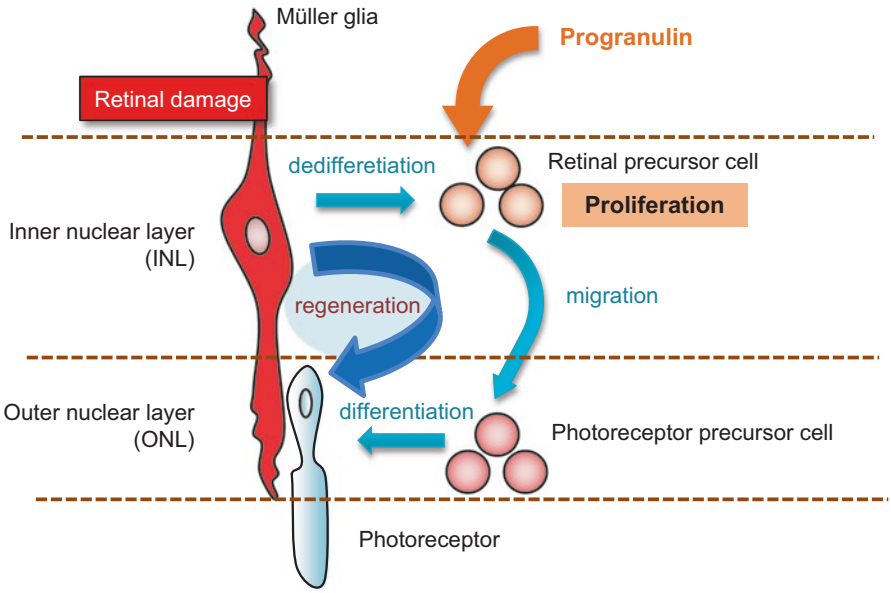

Fig. 2 A schema showing the regenerative effect of progranulin during retinal damage. Retinal damage induces the dedifferentiation of Müller glia, which reprogramming generates retinal precursor cells. Retinal precursor cells are capable of migrating to the outer nuclear layer (ONL) and differentiating into various retinal cells. In addition, progranulin supports retinal precursor cell migration to the ONL and encourages their differentiation to photoreceptor cells

retinal development has been investigated. To determine the effect of progranulin in retinal development, progranulin was combined with a primary retinal cell culture containing precursor cells. Mouse retinas are enucleated at postnatal day 8 (P8). The P8 retina contains immature retinal cells (Swaroop et al. 2010). It is thought that progranulin can promote the differentiation of retinal precursor cells to photoreceptor cells in primary retinal cell culture. Progranulin decreases the number of doublecortin (DCX) and CRX positive retinal precursor and photoreceptor precursor cells in primary retinal cell culture. Progranulin increases the number of rhodopsin positive cells compared to the control group (Kuse et al. 2016). Therefore, progranulin increases the differentiation of photoreceptor cells and results in the decrease of retinal precursor cells. To address the mechanism of progranulin, the phosphorylation of the HGF receptor was investigated. Progranulin treatment increases the phosphorylation of the HGF receptor after 5 min in primary retinal cells. Co-incubation with the HGF receptor inhibitor, SU11274, attenuates the phosphorylation. SU11274 inhibits the increase of rhodopsin positive cells induced by progranulin (Kuse et al. 2016). Progranulin exerts the effect of photoreceptor differentiation via HGF receptor signaling. HGF receptor abundantly exists in the ONL during retinal development; although, the association between this signaling and photoreceptor differentiation remains unknown (Sun et al. 1999). Moreover, the level of photoreceptor cell differentiation and HGF receptor phosphorylation is

investigated using global progranulin-deficient mice (Kayasuga et al. 2007). In progranulin-deficient retina, the phosphorylation of the HGF receptor is inhibited compared to the wild type (WT) retina. The expression of CRX is increased and the rhodopsin expression is decreased in the progranulin-deficient retina at P9 (Kuse et al. 2017). In other words, progranulin deficiency inhibits the maturation of photoreceptors from the photoreceptor precursor cells at P9. These results strongly suggest progranulin promotes the differentiation of photoreceptors during development (Kuse et al. 2016, 2017). These reports suggested that progranulin might progranulin-deficient retina alteration of progranulin be associated with the migration and the proliferation of retinal precursor cells and their differentiation to photoreceptor cells (Fig. 3). At 8–12 weeks old, the progranulin-deficient retina was examined to reveal the impact on abnormal photoreceptor differentiation. The ONL thickness was significantly decreased in the progranulin-deficient retina compared to WT retina (Kuse et al. 2016). The cell number in the ganglion cell layer (GCL) is also decreased in progranulin-deficient retinas. A decrease in rhodopsin expression is observed in progranulin-deficient retina compared to WT retina. These results suggest that progranulin can potentially affect photoreceptor cell development and ONL formation.

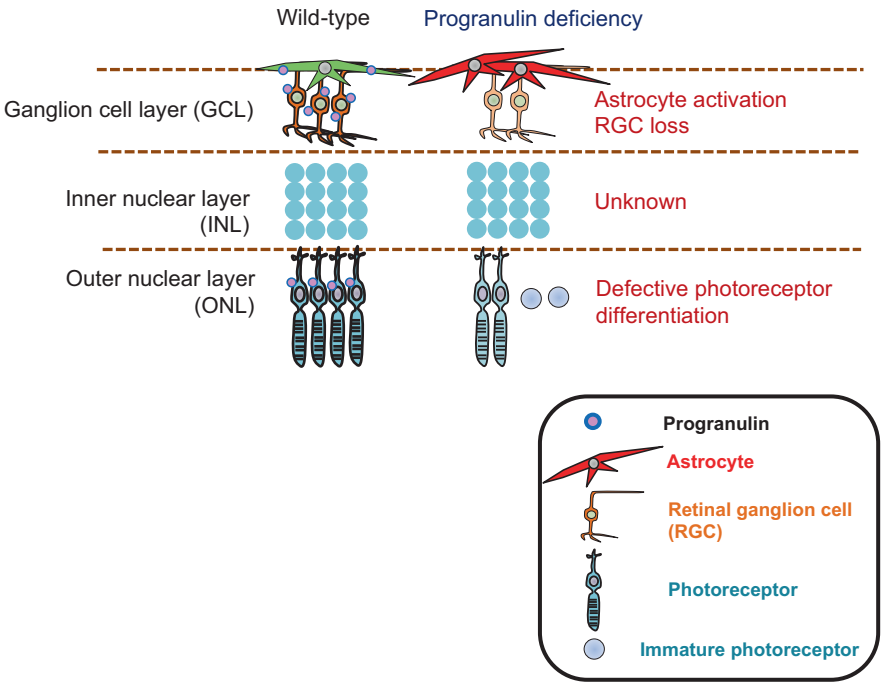

Fig. 3 The alteration of a progranulin-deficient retina. Progranulin is mainly secreted by microglia. In the immature stage, the absence of progranulin results in astrocyte activation and RGC loss. Rather than influence the RGC, retinal progranulindeficiency causes defective photoreceptor differentiation

The Role of Progranulin Regarding Astrocytes

The cell number in the GCL is decreased in adult progranulin-deficient mice, though there is no change in the thickness of the inner plexiform layer (IPL), inner nuclear layer (INL), or outer plexiform layer (OPL). The nuclei of retinal ganglion cells (RGCs) exist in the GCL. RGCs are located in GCL among the astrocytes, and they send their axons toward the optic nerve and to the lateral geniculate nucleus and superior colliculus in the brain (Yonehara et al. 2008). Astrocytes secrete different types of growth factors and cytokines especially when the retina is injured. Astrocytes originate from neural precursor cells and more specifically from astrocyte precursor cells (APCs) (Freeman 2010; Tao and Zhang 2014). In adult mice, astrocytes, microglia, and oligodendrocytes in the optic nerve support the axons of the RGCs (Vecino et al. 2016; Howell et al. 2007). APCs in the retina guide the axons toward the optic nerve during embryonic development (Tao and Zhang 2014), and they are required for the normal development of the synapses of the RGCs (Bialas and Stevens 2013; Clarke and Barres 2013). The RGCs interact with astrocytes. Growth factors such as PDGF-A and sonic hedgehog produced by RGCs promote the migration and proliferation of APCs (Tao and Zhang 2014; Fruttiger et al. 1996; Wallace and Raff 1999). Mature astrocytes play a key role in neurogenesis during development (Song et al. 2002). The changes of RGCs and astrocytes are investigated because a balance of RGCs and astrocytes is important for the normal development of neurons. Brn3a, a specific marker for RGCs is reduced in progranulin-deficient mice (Kuse et al. 2017). GFAP is a marker of activated astrocytes (Krencika and Zhang 2011), and a high expression of GFAP is observed in progranulin-deficient retina compared to WT retina (Kuse et al. 2017). These results demonstrate that progranulin deficiency impairs the survival of RGCs and astrocyte normality. In the optic nerve, phosphorylated neurofilament heavy (p-NFH) and NFH, markers of RGC axons, are accumulated in progranulin-deficient mice (Howell et al. 2007; Lambert et al. 2011). The fluorescent intensity of GFAP is increased in a progranulin-deficient optic nerve. These results confirm the degeneration of the RGCs and the axonal change in the progranulin-deficient optic nerve. While, the expression of GFAP in the hippocampus at CA1, CA3, and dentate gyrus (DG) is not changed between the WT and progranulin-deficient hippocampus (Kuse et al. 2017), which was shown in a previous report using at least 7-months old mice (Ahmed et al. 2010). It is suggested progranulin deficiency may strongly affect the astrocytes in the retina and optic nerve. Taken together, the absence of progranulin can affect the survival of RGCs subsequent to the activation of astrocytes and leads to the immaturity of photoreceptor cells during retinal development.

The Involvement of Progranulin in Retinal Development

The number of RGCs is decreased and astrocytes are activated during development. At P9, the number of Brn3a positive RGCs and the cell number in the GCL are reduced in progranulin-deficient retinas (Kuse et al. 2017). The fluorescent intensities of both astrocyte markers, S100 and GFAP expression, are increased in progranulin-deficient retinas and western blotting shows an increase in GFAP expression (Kuse et al. 2017). The expression of glutamine synthetase (GS), another glial marker, is increased in progranulin-deficient retinas. Therefore, astrocytes and Müller glia are activated. However, there is no change in S100 expression at P1. These results suggest that progranulin deficiency affects the retina in the late postnatal stage (at P9). The phosphorylation of the HGF receptor is reduced in the progranulin-deficient retina at P9 (Kuse et al. 2017), and it is highly likely that progranulin is associated with the HGF receptor (Tsuruma et al. 2014; Li et al. 2010; Kuse et al. 2016). More specifically, it is observed that a decrease of phosphorylation in the progranulin-deficient retina and an increase of the phosphorylation in primary retinal cells by recombinant progranulin are a result of the activity of retinal neuronal cells and not only by astrocytes considering the abundance of astrocytes in the retina. It is reported that *c-met,* an HGF receptor gene, is present in RGCs and exogenous HGF protects RGCs through HGF receptor signaling (Tönges et al. 2011; Wong et al. 2014). Therefore, progranulin may directly contribute to the survival of RGCs through HGF receptor signaling at immature stages. It is reported that excessive activation of astrocytes induces the death of neuronal cells in culture (Olivera-Bravo et al. 2011). Astrocyte markers, S100β, GFAP, and GS are upregulated in progranulin-deficient mice at P9. Most recently, it has been reported that recombinant progranulin directly suppresses the astrocyte-secreted inflammatory cytokines using murine primary astrocytes (Menzel et al. 2017). The excessive activation of astrocytes in progranulin-deficient retina might cause the loss of RGCs. Other reports show the degeneration of RGCs proceed after 12-months old progranulin-deficient mice (Hafler et al. 2014; Ward et al. 2014). Progranulin deficiency causes the accumulation of autofluorescent materials and the mislocalization of TDP-43 in aged mice. The mechanism of degeneration of RGCs could be different between young and aged progranulin-deficient mice.

To clarify the contribution of progranulin to retinal normality, the expression pattern and level were investigated. WT retinas at P9 show the strong expression of progranulin at P1 in WT retinas (Kuse et al. 2017). Immunostaining shows S100 positive astrocytes are partly co-localized with progranulin in P9 WT retinas, but not with P1 WT retinas. Iba-1 positive microglia are almost fully co-localized with progranulin in P1 and P9 retina. In normal development, progranulin expression is increased from P1 to P9. RGCs and astrocytes strongly depend on the presence of progranulin during this stage. An expression of progranulin is also observed in the microglia in optic nerve. Previous reports show that progranulin is mainly secreted by microglia and neurons in the brain during early and adult ages (Petkau et al. 2010; Lü et al. 2013; Kanazawa et al. 2015). Although S100β positive astrocytes are

co-localized with progranulin in the retina and optic nerve, it is generally not observed in the brain astrocytes. This difference between retina and brain could contribute the specificity of effect of retinal astrocytes by progranulin.

Conclusion and Future Perspective

The association of progranulin with pathology is mainly reported in aged mice, and, in particular, lysosomal dysfunction in the CNS (Klein et al. 2017; Tanaka et al. 2014; Zhou et al. 2017; Matsuwaki et al. 2011). The role in juvenile and young stages also remains unrecognized although the expression was observed several tissues in a 2003 report (Daniel et al. 2003). In the area of ophthalmology, there is gradually accumulating evidence that progranulin has important physiological roles.

The major conclusion is that progranulin has a protective effect on the retina and optic nerve. These important findings may help in the development of a neuroprotective drug that targets progranulin. Recent reports show that progranulin can be associated with retinal regeneration, development, and muscle regeneration (Kuse et al. 2016, 2017; Li et al. 2013). Based on our data, progranulin is strongly correlated with the survival of RGCs and the development of the retina (Kuse et al. 2017). In addition, we revealed that PGRN might be associated with the migration and proliferation of retinal precursor cells and their differentiation to photoreceptor cells (Kuse et al. 2016). These results indicate that progranulin could become a key factor in retinal regeneration. In the future, we hope that a treatment targeting progranulin will prevent blindness from retinal degenerative diseases by both neuroprotection and regeneration.

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