Gain And Loss Of Progranulin Have Opposite Effects On Autophagy

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Ph.D. Thesis

GAIN AND LOSS OF PROGRANULIN HAVE OPPOSITE EFFECTS ON AUTOPHAGY

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Abstract

Progranulin (PGRN) is a widely expressed cysteine-rich, secreted glycoprotein with growth factor-like properties. PGRN is involved in both metabolism and neurodegeneration. It has recently emerged as an important regulatory adipokine of glucose metabolism and insulin sensitivity. Individuals with obesity and type 2 diabetes were shown to have increased serum PGRN levels. Heterozygous loss of function mutations in the gene encoding PGRN (GRN) have been described as the major cause of frontotemporal lobar degeneration (FTLD), the most common type of dementia after Alzheimer’s disease (AD), whereas homozygous mutations result in neuronal ceroid lipofuscinosis (NCL), an infantile onset disease. During my Ph.D thesis, I found that hepatic PGRN overexpression in mice results in autophagy impairment leading to metabolic syndrome whereas PGRN loss in mouse brains results in increased activation of autophagy through TNF receptor (TNFR) over-stimulation and C-Jun N-terminal kinase (JNK) activation. Excessive activation of brain autophagy is associated to neurodegeneration and either JNK or autophagy inhibition improve neuronal viability and dysfunction in PGRN deficient neurons and PGRN null C. elegans. Moreover, I found that gain and loss of PGRN have opposite effects on insulin-like growth factor 1 (IGF-1) level, implicated in both metabolism and neurodegenerative disorders. IGF-1 has been linked also to autophagy in several studies. Activation of IGF-1/insulin receptor substrate 2 (IRS-2) signaling, that has been previously demonstrated to activate autophagy, was found in PGRN deficient brain, thus suggesting a role for IGF-1 in the enhanced autophagy associated to neurodegeneration induced by PGRN loss. As stated above, FTLD is the most common form of early-onset dementia, after AD. However, how PGRN loss causes neurodegeneration is not fully understood. These results unravel novel mechanisms
involved in neurodegeneration induced by PGRN loss and open new avenues for the investigation of new therapeutic studies in FTDL.
## List of abbreviations

- **PGRN**, Progranulin
- **FTLD**, Frontotemporal Lobar Degeneration
- **AD**, Alzheimer's disease
- **NCL**, Neuronal Ceroid Lipofuscinosis
- **TNFR**, tumor necrosis factor α receptor
- **JNK**, C-Jun N-terminal kinase
- **IGF-1**, insulin-like growth factor 1
- **IRS-2**, insulin receptor substrate 2
- **SLP1**, secretory leukocyte protease inhibitor
- **HDL**, high density lipoprotein
- **CNS**, central nervous system
- **MAPK**, mitogen activated protein kinase
- **ERK**, extracellular regulated kinase
- **PI3K**, phosphatidylinositol-3-kinase
- **TEFb**, transcription elongation factor b
- **SORT1**, sortilin1
- **Vps10**, vacuolar sorting protein 10
TNF-α, tumor necrosis α

T2D, type 2 diabetes

BMI, body mass index

IL-6, interleukine 6

HFD, high-fat diet

WT, wild-type

IRS-1, insulin receptor substrate 1

ALS, amyotrophic lateral sclerosis

TDP43, transactive response DNA binding protein with a molecular weight of 43 kDa

GWAS, genome-wide association studies

SNPs, single nucleotide polymorphisms

TFEB, transcription factor-EB

CHMP2B, charged multivesicular body 2B

VCP, valosin-containing protein

LDs, lipid droplets

TGs, triglycerides

PD, Parkinson’s disease

HD, Huntington’s disease
BECN1, Beclin1

ACD, autophagy cell death

SAPKs, stress activated protein kinases,

Bax, Bcl-2 associated X protein

Bcl-2, B cell lymphoma 2

TRAF2, TNF receptor activated factor 2

TRADD, TNF receptor associated death domain

HDAd, helper-dependent adenoviral vector

AFP, α-fetoprotein

PFA, paraformaldehyde

PBS, phosphate buffered saline

TBST, tris buffered saline tween

PI3K III, phosphatidylinositol 3-Kinase class III

3-MA, 3-methyladenine

LDH, lactate dehydrogenase
Introduction

**PGRN: cellular and physiologic functions**

The Progranulin (PGRN) protein is encoded by the *GRN* gene composed of 13 exons, although exon 1 is non-coding. PGRN is a secreted glycoprotein with growth factor like properties, consisting in 7.5 tandem repeats of a 12-cysteine module called granulin or epithelin domain separated by linker regions. The 12-cysteine motif forms six disulfide bridges with the peptide backbone adopting an unique three dimensional structure of a parallel stack of beta hairpin [1]. PGRN is synthesized as a 68.5 kDa protein that is N-glycosylated to become a mature secreted 88 kDa protein [2]. Secreted PGRN is cleaved into 6 kDa granulin peptides, each composed of cysteine repeat motifs. Cleavage of PGRN occurs at the intra-linker spacer regions by several proteases including elastase [3], proteinase [4], matrix metalloproteinase-14 [5] and ADAM metallopeptidase with thrombospondin type 1 motif [6]. PGRN cleavage is inhibited by secretory leukocyte protease inhibitor (SLPI) [3] and high-density lipoprotein (HDL)/Apolipoprotein A-I complex [7].

*GRN* is expressed in several tissues showing a prominent expression in epithelial, immune and, neuronal cells [8]. In particular a strong expression of GRN mRNA was found in highly proliferative epithelia including those of the intestinal crypt, skin, and reproductive tracts [8]. In contrast *GRN* shows low expression in less proliferative and highly differentiated cells such as those of alveolar lung and renal tube epithelia [8]. Moreover, *GRN* shows very marked expression in pheripheral immune system tissues such as spleen and lymph nodes. [8]. The sequence of human *GRN* was indeed first isolated
from mRNA derived from bone marrow cells [1]. *GRN* is also highly expressed in the central nervous system (CNS) with a similar dual pattern of expression: neurons and neuroinflammatory cells. PGRN has been detected in neurons of the neocortex, hippocampus, arcuate nucleus and ventromedial hypothalamus, Purkinje cells of the cerebellum and in motor neurons [8]. In the CNS, PGRN can function as neurotrophic growth factor increasing cell viability [9] and neurite outgrowth [10]. PGRN knock-down in rat hippocampal neurons results in decreased neuronal arborization and length and synaptic densities [11] showing its role in neuronal morphology and establishing inter-neuron connections.

PGRN regulates several cellular functions *via* different signaling transduction pathways. It is involved in cell growth and survival activating the two main mitogenic signaling pathways. In fact, PGRN stimulates the phosphorylation of p44/42 MAPK (mitogen activated protein kinase) in the ERK (extracellular regulated kinase) signaling pathway, and PI3K (phosphatidylinositol-3- kinase), AKT/protein kinase B, and p70S6 kinase in the PI3K pathway [12]. The correlation between *GRN* expression and epithelial tissue proliferation that was noted for adult tissues, clearly extends to the earliest stages of life. *GRN* is strongly expressed in oocytes [13]. After fertilization *GRN* expression declines to the 4 cell stage and it is re-expressed in 8 cell embryo achieving highest pre-implantation levels in the trophectoderm of the blastocysts [13]. Adding PGRN to 8 cell embryos accelerates cavitation, and the expansion of the blastocoel, and acts as a growth factor for trophectodermal cells [13]. Blocking the endogenously secreted PGRN with an immunoneutralizing antiserum significantly delayed the formation of blastocysts demonstrating that PGRN is required for the critical transition from the morula to the blastocyst that is essential for embryogenesis [13].
Moreover, it has been found that PGRN plays an important role in inflammation as an antinflammatory protein affecting the TNFR receptor signaling.

In addition to the ability of secreted PGRN to regulate several cellular functions via signal transduction pathways outside the cells, it has been demonstrated that intracellular PGRN can act as a repressor of transcription from cellular promoters [14]. In particular, PGRN is able to directly bind and inhibit the transcription elongation factor b (TEFb) that is required for the transcription from several cellular promoters [14].

Secreted PGRN explicates many of its biological functions by binding to its receptors. The first identified PGRN receptor was sortilin-1 (SORT1) [15] a neuronal transmembrane receptor[16]. SORT1 is a member of the vacuolar protein sorting 10 (Vps10) family and is a lysosomal sorting receptor responsible for the delivery of extracellular prosaposin and intracellular Golgi-derived cathepsins to lysosomes [17]. Interestingly, SORT1 was previously identified as receptor for other neurotrophic factors, supporting the role of PGRN in neuroprotection [18]. SORT1 is expressed primarily by neurons, and has minimal expression in microglia. PGRN has a high affinity for SORT1 and, upon binding to SORT1 on neuronal cells surface, rapidly undergoes endocytosis and delivery to lysosomes. Therefore, SORT1 regulates the extracellular level of PGRN in the brain.

More recently, tumor necrosis factor receptors (TNFR) have been identified as receptors of PGRN [19]. PGRN binds and antagonizes both TNFR1 and TNFR2 inhibiting TNFα signaling pathway in a dose-dependent manner [19]. The TNFα signaling pathway is the major mediator of inflammation and it is involved in different human diseases including diabetes and rheumatoid arthritis. It has been demonstrated that depletion of GRN induces a more severe arthritis in TNFα transgenic mice and in mouse model of
cartilage-induced arthritis. In contrast, the administration of murine recombinant PGRN totally reverts the signs of arthritis in these mouse models [19]. Based on the recognized anti-inflammatory effect of PGRN, novel therapeutics against inflammatory conditions, such as rheumatoid arthritis, are under development [19].

**PGRN and metabolism**
PGRN is emerging as an important regulatory adipokine of glucose metabolism and insulin sensitivity. Individuals with visceral obesity and type 2 diabetes (T2D) were shown to have increased PGRN serum levels [20]. Additionally, circulating PGRN levels are positively correlated with body mass index (BMI), fat mass, fasting glucose, insulin levels and insulin resistance, the hallmark of T2D [20, 21].

Several studies have been performed in order to elucidate the association between PGRN and metabolic diseases [22]. Elevated levels of PGRN were found in the white adipose tissue of a well-characterized mouse model of obesity (ob/ob mice) [22]. The administration of murine recombinant PGRN to wild-type mice induced an increase of serum insulin level, increased fat mass, insulin resistance and elevated levels of interleukin 6 (IL-6) that is strongly involved in the development of insulin resistance [22]. Therefore, PGRN mediates insulin resistance *in vivo* inducing IL-6 expression in adipose tissue. Interestingly, *Grn* knock-out mice upon high-fat diet (HFD) showed reduced body weight, fasting insulin and fat mass compared to wild-type (WT) mice consuming HFD thus indicating that PGRN loss prevents HFD-induced insulin resistance *in vivo* [22]. Moreover, insulin resistance, that was found to be induced by repeated injections of recombinant murine PGRN, was prevented by the administration of anti-IL-6 neutralizing antibodies [22]. PGRN might also promote insulin resistance by directly inhibiting the insulin-
signaling cascade. Treating 3T3-L1 adipocytes with exogenous PGRN inhibited insulin-induced phosphorylation of insulin receptor substrate (IRS)-1 and AKT in a dose-dependent manner, while knockdown of PGRN in 3T3-L1 adipocytes enhanced insulin sensitivity [22]. The phosphorylation of insulin receptor in response to insulin was not affected, suggesting that PGRN inhibits insulin signaling at an early step between insulin receptor phosphorylation and IRS-1 phosphorylation.

Although the role of PGRN on metabolism has been recognized, the mechanisms underlying PGRN-mediated insulin resistance are still not well understood. Recently, it has been found that short-term treatment with recombinant PGRN induces insulin resistance both in murine liver and hepatocytes and this was associated to defective autophagy [23]. The relationship between autophagy and PGRN was attributed to the involvement of a variety of adipocytokines including IL-6, and TNFα [24]. Interestingly PGRN-induced insulin resistance and defective autophagy in liver was found to be reverted by the administration of a TNFR blocking peptide thus indicating that PGRN mediates insulin resistance and impaired autophagy via TNFR signaling in the liver. Infact, mice treated with recombinant PGRN showed a strong activation of NFKB, a well known downstream target of TNFR [23]. These evidences were in contrast with the inhibitory effect of PGRN on TNFR pathway that was observed in mouse model of cartilage-induced arthritis [19]. Therefore, the mechanisms by which PGRN mediates insulin resistance remain elusive and need to be further elucidated.

**PGRN and neurodegeneration**

Heterozygous mutations in the GRN gene encoding PGRN are responsible for frontotemporal lobar dementia (FTLD), the most common cause of dementia before 65
years of age after Alzheimer’s disease (AD) [25]. Homozygous GRN mutations instead are associated to neuronal ceroid lipofuscinosis (NCL) [26], a severe neurodegenerative disorder presenting with juvenile onset of progressive degeneration of the brain and retina, with accelerated intracellular accumulation of lipofuscin [27].

FTLD patients develop severe atrophy of the frontal and temporal lobes, with behavioral changes [28]. Some patients also display motor neuron impairments resembling amyotrophic lateral sclerosis (ALS) [29]. Neuropathological hallmarks of PGRN deficient brains are dystrophic neurites, inflammation, neuronal and microglial ubiquitin-positive inclusions, and severe neuronal loss mainly affecting the frontal and temporal lobes but also found in the striatum, thalamus, substantia nigra, and hippocampus [30]. On autopsy, brains of patients with GRN mutations show accumulation of the intracellular transactive response DNA binding protein with a molecular weight of 43 kDa (TDP-43) [31]. TDP-43 is an RNA/DNA binding protein with multiple roles in RNA metabolism and transcriptional repression. In FTLD-TDP patients, TDP-43 is hyperphosphorylated, cleaved into C-terminal fragments, and translocates from the nucleus to the cytoplasm [31]. However, the relationship between PGRN and TDP-43 remains to be investigated.

A large number of GRN mutations have been identified in FTLD patients [32] and most of them lead to premature stop codons, as a result of nonsense mutations or frameshifts. Therefore haploinsufficiency is responsible for the development of FTLD [33].

The lysosomal transmembrane glycoprotein TMEM106b has been found to be a genetic modifier of PGRN expression. Genome-wide association studies (GWAS) found single nucleotide polymorphisms (SNPs) in or near TMEM106b to affect the age of onset of FTLD [34]. Increased levels of TMEM106B are associated to enlarge lysosomes and
impaired endolysosomal trafficking [35]. Moreover, exogenous expression of TMEM106B increases PGRN levels, possibly due to its regulation of the endolysosomal pathway [35]. Both PGRN and TMEM106B are localized into lysosomes and PGRN has been found related to lysosomal function [36]. In fact, it has been previously found that PGRN overexpression increases the levels of lysosomal proteins, LAMP1 and LAMP2, and lysosomal size in human cells [36]. Moreover, it has been found that PGRN is a target of transcription factor-EB (TFEB) [36, 37] that controls most of the known lysosomal genes [37]. Two binding sites for TFEB have been identified upstream of the GRN coding sequence and the overexpression of TFEB in human cells results in a strong increase of GRN mRNA levels [36].

Altered vesicle trafficking and protein homeostasis appears be important to FTLD pathogenesis, as shown by mutations in two other genes more rarely responsible for FTLD [26]: charged multivesicular body 2B (CHMP2B) and valosin-containing protein (VCP). CHMP2B encodes a subunit of the ESCRTIII complex involved in endosomal trafficking and degradation, and mutations result in impaired fusion of endosomes with lysosomes [38]. VCP is an ATPase with multiple functions, including endoplasmic reticulum-associated degradation of misfolded proteins. VCP mutations result in vacuolization and impaired protein degradation [39].

Taken together, these evidences suggest that alterations in autophagy-lysosomal pathway are involved in the pathogenesis of FTLD. Moreover, because the genetic defects responsible for FTLD converge to the same biological pathways, a common therapeutic strategy may be used for therapy.
In addition, complement activation and microglia-mediated synaptic pruning have been recently found to play a role in neurodegeneration caused by PGRN deficiency [40]. Nevertheless, the mechanisms underlying neurodegeneration are still not well understood.

Constitutive Grn knock-out mice (Grn-/-) [41] are viable and are born with normal Mendelian frequencies, although increased mortality was observed beginning at 11 months of age. Beginning at 6 months of age, Grn knock-out mice show an age dependent activation of astrocytes and microglia and increased brain ubiquitination and accumulation of lipofuscin [41]. Additionally, aging mice show increase phosphorylation of TDP43 that maintains its predominant nuclear localization and is not present in cytoplasmic ubiquitin positive inclusions [41]. Increased IGF-1 level has been found in brains of Grn-/-mice [41]. IGF-1 signaling has been associated with decreased longevity and early onset of age-related disorders in diverse species [42]. Therefore, disturbances of the IGF-1 axis could be an important mechanism responsible for the increased cellular ageing observed in Grn-/- animals. Moderate abnormalities in anxiety-related behaviours, social interactions, motor coordination, and novel object recognition were observed in 8-month-old Grn-/- mice [41]. Additionally, altered synaptic connectivity and impaired synaptic plasticity were reported in 10- to 12-month-old Grn-/- mice [41].

Grn-/-mice are protected from HFD induced obesity and insulin resistance [22]. In addition, Grn-/-mice have increased susceptibility to collagen-induced arthritis [19], contact dermatitis and infections [43], providing evidence of multiple deficiencies in the immune response. In summary, Grn-/- mice recapitulate several features of FTLD patients, and thus represent a good tool to investigate the role of PGRN in neurodegeneration.
**Autophagy and its role in metabolism and neurodegeneration**

Autophagy is a catabolic process that degrades the cytosolic components, such as proteins and organelles, by transporting them to the lysosomes. Briefly, autophagy proceeds through the capture of portions of cytoplasm containing target material inside expanding membranes, which finally enclose to form double-membrane vesicles called autophagosomes. Fully formed autophagosomes are shuttled along microtubules to lysosomes, where fusion and degradation occur [44]. This removal and recycling serves as an emergency energy supply during nutrient deprivation, but autophagy has also be linked to other protective functions such as capture of invading pathogens [45], context dependent tumor suppressive and tumorigenic qualities [46], the removal of toxic aggregate-prone proteins often linked to neurodegeneration [47] and lipid homeostasis [48]. In particular, autophagy has been shown to play an important role in the turnover of hepatic lipid droplets (LDs). Intracellular lipids, stored in LDs, are essential to cells as an energy source, structural components for membranes, synthetic building blocks for other molecules, such as hormones, and as mediators of cell signaling. The ability to safely store adequate, but not excessive, amounts of lipids and to metabolize them when needed is critical for cell function and survival. Normally, LDs are metabolized by cytoplasmatic neutral hydrolases to supply lipids for cell use. Recently, autophagy (lipophagy) has been identified as alternative pathway of lipid degradation. Lipophagy was initially described in hepatocytes, which become a major site of excessive lipid accumulation in obesity and metabolic syndrome [49]. A pharmacological or genetic inhibition of autophagy in cultured hepatocytes increased cellular triglycerides (TG) content and LDs number and size in response to a lipid challenge [48]. These in vitro findings were confirmed in mice with a hepatocyte-specific knockout of a gene essential for autophagosome formation, *Atg7* [50]. Liver-specific *Atg7* knock-out mice displayed large livers that are accumulating cellular
debris, including damaged organelles, ubiquitinated proteins, and a significant increase in lipid content [48]. In addition, overexpression of TFEB leads to an amelioration of obesity phenotype observed in mice fed with HFD [51]. Therefore, autophagy represents an alternative mechanism for the maintenance of lipid homeostasis and its manipulation could represent a therapeutic approach for metabolic diseases.

Moreover, several studies have firmly established the importance of autophagy in both normal functioning and patho-physiological conditions of the brain. Neurons have highly specialized structures for intercellular communication, which typically include the axon, dendrites and synapses. Synapses represent region of high-energy demand and protein turnover; they contain abundant mitochondria which make them more susceptible to the consequences of dysfunctional autophagy. In addition, the fact that neurons are postmitotic and do not replicate in general might predispose them to accumulate toxic proteins and damaged organelles inside neurons that could become diluted through cell division in replicating cells. For these reasons neuronal integrity is more sensitive to alterations in basal autophagy than that of non-neuronal cells. Impairments in the quality control of proteins and organelles likely disrupt normal neuron function due to the accumulation of toxic protein aggregates, ultimately leading to neurodegenerative conditions observed in diseases such as AD, Parkinson’s disease (PD) and Huntington’s disease (HD). Recent experimental evidences indicate that autophagy has a neuroprotective role by facilitating clearance of misfolded proteins and aggregates. In the APP transgenic mouse model of AD the reduced expression of the autophagic protein, Beclin1 (BECN1), results in an increase of intraneural accumulation of toxic proteins which was ameliorated by the lentiviral expression of BECN1 [52]. The pharmacological inhibition of MTOR, a
well known inhibitor of autophagy, with rapamycin rescued cognitive deficits of APP mice by increasing autophagy [53].

Although neuronal autophagy appears primarily to be a protective process in the nervous system, it can also play a paradoxical role in neuronal death. Under certain pathophysiological conditions or in absence of tight regulation, autophagy may exceed a crucial threshold, causing the catabolism of cytoplasmic factors, regulatory molecules and organelles that are essential for survival. Autophagic cell death (ACD) is characterized by the presence of abundant autophagosomes in the dying cell resulting in extensive cytoplasmic vacuolization [54]. This has largely been attributed to increases in autophagosomes synthesis and flux, causing excessive degradation of important cell components [54]. It has been reported that activation of autophagy by the autophagy-inducing peptide Tat-Beclin-1 can cause cell death with unique morphological features of autophagy. This type of cell death is blocked by either pharmacological or genetic inhibition of autophagy, but not by impairment of known regulators of either apoptosis or necroptosis [55]. Oxidative stress, the major pathogenic mechanism of PD, has been shown to increase ACD in dopaminergic neurons of PD mice and the treatment with rapamycin potentiates cell death induced by oxidative stress [56]. In neonatal mice, neuron-specific deletion of Atg7 protects against cerebral hypoxia–ischemia-induced hippocampal neuron death [57], and in adult rats shRNA targeting BECN1 prevents neuronal death in the thalamus following focal cerebral infarction [58]. Interestingly, increased autophagy is also thought to be responsible for FTLD due to mutations of CHMP2B gene encoding a component of the endosomal ESCRTIII complex [59] and autophagy inhibition had ameliorative effects [60].
Despite the considerable recent advances, the understanding of the dual role of autophagy in cell survival and cell death remains incomplete. It is still not fully understood what factors determine whether autophagy is cytoprotective or cytotoxic and how autophagy contributes to death. It is likely that the dual role of autophagy in cell death is context-dependent.

Therefore, an improved understanding of neuronal autophagy will provide novel insights into the pathogenetic mechanism of dysfunctional autophagy that underlie common neurodegenerative diseases and, ultimately, help develop therapeutic interventions for autophagic dysregulation.

**MAPK/JNK signaling and autophagy**

The MAPK signal transduction is one of the most important regulatory mechanisms in eukaryotic cells. Six MAPK sub-families have been identified in mammalian cells; they are JNK1/2/3, ERK1/2, p38MAPK (p38 α/β/γ/δ), ERK7/8, ERK3/4 and ERK5/BMK1 (big MAP kinase 1) [61]. After activation by upstream kinases, different subfamilies regulate various physiological processes in cells, including inflammation, stress, cell growth, cell development, differentiation and death, through multiple substrates like transcription factors [62]. JNKs were initially identified as the stress-activated protein kinases (SAPKs) in the mouse liver treated with cycloheximide to induce inflammation and apoptosis [63]. There are three genes that encode JNKs in mammals: JNK1, JNK2 and JNK3, among these, JNK1 and JNK2 are widely expressed in vivo whereas JNK3 is expressed in the brain, heart and testis. JNKs are activated by a number of stressors, including UV irradiation and oxidative stress, which can induce apoptosis or growth inhibition [64]. MKK4 and MKK7, the upstream kinases (namely, MAP2K) of JNK pathway, are activated
by different upstream MAP3K respectively [62]. Once activated, JNK translocates from cytoplasm to nucleus [65]. The downstream targets of JNK include the transcription factor c-Jun, which translocates to the nucleus after JNK mediated phosphorylation. c-Jun is best known to regulate expression of pro-apoptotic or antiapoptotic genes \textit{Bax} (Bcl2-associated X protein) and \textit{Bcl-2} (B cell lymphoma 2) [66]. When activated, JNK phosphorylates serine residues 63 and 73 at c-Jun N-terminal thus activating c-Jun and enhancing its transcriptional activity [62].

JNK signaling pathway is activated by several stimuli. One of these is TNF\textalpha [67]. TNF\textalpha binds TNFR inducing receptor aggregation followed by the recruitment of different cytoplasmic signaling proteins [68]. One of these is TNF receptor activated factor 2 (TRAF2) that is recruited to TNFR via its interaction with TNF receptor associated death domain (TRADD) [69]. Recruitment of TRAF2 is followed by sequential activation of several MAPK that in turn activate JNK and NFK\beta pathway [69].

Several studies have shown that JNK can modulate autophagy at multiple regulatory levels. It has been demonstrated that JNK induces the expression of multiple \textit{ATG} genes in presence of several stressors such as oxidative stress [70], endoplasmic reticulum stress [71] and exposure to TNF\textalpha [72]. Moreover, JNK indirectly activates autophagy by phosphorylation of \textit{FOX} (forkhead box) O transcription factor FoxO. Oxidative stress leads to phosphorylation of FoxO \textit{via} small GTPase Ral-activating JNK, thus inducing FoxO nuclear localization and increases its activity [73]. FoxO transcription factors can regulate autophagy. It has been proposed that cytoplasmic FoxO transcription factor is necessary for inducing autophagy. Down-regulation of cytoplasmic FoxO inhibits p62 degradation and LC3 II accumulation, resulting in autophagosomes decrease [74]. It has also been reported that FoxO induces the expression of \textit{ATG} genes such as \textit{LC3, BNIP3}
and *ULK1 in vivo*, during starvation [75]. Therefore, JNK induces autophagy by activation of FoxO factor. The anti-apoptotic protein Bcl-2 and other Bcl family members, such as Bcl-XL can inhibit autophagy [76] Bcl-2/Bcl-XL combines with BECN1 through anchoring groove in the BH3 domain; this interaction is lost when autophagy-promoting signaling occurs [76]. Under starvation condition JNK is activated and phosphorylates Bcl-2 at aminoacid residues (Thr69, Ser70 and Ser87) disrupting the interaction BECN1-Bcl-2 inducing the activation of PI3K-Vps34-BECN1 complex thus inducing autophagy [76]. In addition, JNK can promote autophagy via p53 phosphorylation [77] resulting in upregulation of pro-autophagy genes [78]. These evidences indicate that JNK can regulate autophagy in different ways.

JNK participates in multiple stimulation-induced autophagic events, including endoplasmic reticulum stress [71] caspase inhibition [79] and IGF-1 treatment [72]. Moreover, JNK has been related to ACD. The activation of JNK pathway, and c-Jun selective phosphorylation were found to be involved in autphagic hippocampal neurons death [80]. Indeed, neuronal cell death is neither apoptosis nor necrosis but is in fact characteristic of autophagic neuronal death. This is inhibited by the cell-permeable inhibitors of JNK and its downstream substrates [80]. The administration of SP600125, a specific inhibitor of JNK activation, has been found to show beneficial effects in mouse model of AD[81]. Increased autophagy has been found in hippocampus of AD mice and the intraperitoneally injection of SP600125 leads to the normalization of autophagy levels associated to the improvement of memory deficit [81]. Taken together, these evidences indicate that JNK signaling pathway is an important mediator of ACD and could represent a therapeutic target for pathological conditions in which ACD is implicated.
Aims of the project

PGRN is a protein with growth factor-like properties regulating several biological functions. Interestingly, PGRN has been recently found to be strongly involved in both metabolism and neurodegeneration.

In particular, elevated levels of PGRN have been associated to metabolic diseases such as type 2 diabetes and obesity [22]. To elucidate the link between PGRN and these metabolic diseases, during my PhD training I investigated the role of PGRN overexpression in vivo by injecting WT mice with a helper-dependent adenoviral vector harboring the human GRN gene under the control of a liver-specific promoter. Once assessed the phenotypic alterations due to PGRN overexpression, I investigated the mechanisms underlying these changes. Based on my previous evidences that PGRN overexpression induces an increase of lysosomal size and lysosomal proteins levels in human cells [36] and considering the important role of the autophagic-lysosomal pathway on liver metabolism, I investigated the effects of PGRN overexpression on autophagy. To do that I first performed in vitro studies by generating a human cell line stably overexpressing the GRN gene then, I analyzed the autophagy flux in vivo in PGRN overexpressed livers. These results allowed me to establish a link between PGRN-induced metabolic alterations and autophagy.

Heterozygous loss-of-function mutations in the GRN gene are responsible for FTLD, a common type of early onset dementia [25]. The mechanisms underlying neurodegeneration due to PGRN deficiency are still not fully understood. Several studies have shown that autophagy is involved in many neurodegenerative disorders [52]. Based
on this observation, I analysed, both in vitro and in vivo, the autophagic flux in GRN deficient models.

Autophagy is generally viewed as a neuroprotective mechanism in different neurodegenerative diseases [52]. However unrestrained autophagy has been associated to neuronal cell death [54]. Therefore, during my PhD training I evaluated the role of autophagy on the neurodegenerative phenotype associated to PGRN deficiency. In order to achieve this objective I modulated autophagy in both in vitro and in vivo PGRN-deficient systems by using well-known autophagy inhibitors. The results obtained through the experimental plan I followed allowed me to investigate the involvement of autophagy in the pathogenesis of FTLD.
Materials and Methods

**Mouse studies.** Animal procedures were performed in accordance to regulations of the Italian Ministry of Health. Wild-type C57BL/6 mice were all males and were purchased from Charles-River. Colonies of GFP-LC3 transgenic [82] and Grn-/ mice (purchased from Jackson laboratory) were maintained at the IGB-TIGEM animal facility. For vector injections, I injected male 8-week-old mice were injected intravenously (retro-orbital) with helper-dependent adenoviral (HDAd) vectors (HDAd-AFP or HDAd-PGRN) at the dose of $1 \times 10^{13}$ viral particles (vp)/kg of body weight. Mice were fed ad libitum with standard diet or high-fat diet containing 21% total lipid (42% calories as anhydrous milk fat; PF4100, Mucedola). Body weights of injected mice were measured to the nearest 0.1 g monthly until sacrifice. For each mouse, average daily food intakes were calculated for 4 consecutive days by weighing the food and the calculated intakes were adjusted for body weight[83]. Serum samples of injected mice were collected by retro-orbital bleedings before and 4, 12, 24, 48 weeks after vector injections. Serum PGRN and AFP levels were measured by Quantikine ELISA kit for mouse/rat PGRN and mouse AFP (R&D System), respectively. Adipokine profile was performed by mouse Adipokine Array Kit (R&D System) on mouse sera collected at 4 weeks post-vector injection. Serum IGF-1 levels were measured in 12 week-old Grn-/ and age- and gender-matched wild-type controls by mouse/rat IGF-1 Quantikine ELISA kit (R&D System). Measurements of cholesterol (BioAssay System), triglycerides (BioAssay System), and free glycerol (Abcam) was performed at 24 weeks post-injection. For glucose tolerance test, vector-injected mice were injected intraperitoneally with glucose (2mg/g of body weight) after 12 hours of fasting and blood samples were collected through tail bleedings at baseline, 15, 30, 60, 120 and
180 minutes post-glucose injection for glucometer (FreeStyle Lite) measurements. Prior to sacrifice, mice were starved for 24 hours and then transcardially perfused with phosphate buffered saline (PBS). Weights of liver and epididymal fat were measured at 48 weeks post-vector injection mice consuming regular diet and 24 weeks post-vector injections for mice consuming HFD. Livers were fixed in 4% paraformaldehyde (PFA) overnight and immersed in 30% sucrose in PBS overnight for cryoprotection. Brains of 12 week-old of age wild-type and Grn/- mice were homogenized using Tissue Lyser LT (Qiagen) in PBS.

**HDAd vectors.** The HDAd-PGRN and HDAd-AFP vectors contain the following elements (from 5’ to 3’): a liver restricted phosphoenolpyruvate carboxykinase promoter, the ApoA1 intron, the human PGRN and AFP cDNA, the woodchuck hepatitis virus post-transcriptional regulatory element, the ApoE locus control region and the human growth hormone poly(A)[84]. HDAd vectors were produced in 116 cells with the Helper virus AdNG163 as previously described [85, 86]. The viral vectors were generated by TIGEM core for the production of HDAds.

**Western Blots.** Cells and tissues were homogenized in RIPA lysis buffer [50 mM Tris base pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% SDS] containing protease inhibitors cocktail (Complete Mini, Roche) and phosphatase inhibitors cocktail tablets (Roche) according to manufacturer’s instructions. Lysates were cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C. Proteins were quantified using DC Protein Assay kit (Bio-Rad). After dilution in buffer [0.125 M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 1M DTT], proteins were incubated at 99°C for 5 minutes. Protein extracts were
resolved by SDS polyacrylamide gel electrophoresis and proteins were transferred to an Immobilon-P PDVF membrane (Millipore), which was then blocked in 5% nonfat dry milk (Bio-Rad) in TBST buffer (1X Tris buffered saline, 0.1% Tween-20) for 1 hour. Primary antibodies used for immunoblots are listed in Table 1. Membranes were washed with TBST buffer and incubated with a peroxidase-linked donkey anti-rabbit IgG and anti-mouse IgG antibody (GE Healthcare). β-actin and GAPDH were evaluated using a mouse monoclonal antibody (Novus Biological) and a peroxidase-linked donkey anti-mouse IgG secondary (GE Healthcare). Proteins were detected with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and ChemiDoc Imaging System (UVP, Cambridge, UK). The digitized images were analyzed by using Quantity One 4.6.6 software (Bio-Rad).

The immunoprecipitation for IRS-2 was performed using brain lysates in RIPA buffer. Anti-IRS-2 antibody (5μg) (Table.1) was added to lysates and was incubated overnight at 4°C in rotation. Beads (Dynabeads®, Thermo Fishers Scientific) were added and incubated 3 hours at 4°C in rotation. The immune complex was isolated using DynaMag™-2 magnet (ThermoFisher scientific) and then washed three times with 0.02%Tween PBS. The immune complex was eluted with sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and 1M DTT), boiled for 5 min, and then recovered using DynaMag™-2 magnet (ThermoFisher scientific). The eluates were resolved in SDS-polyacrylamide gel electrophoresis.

Stainings and immunofluorescence. For GFP-LC3 puncta analysis, frozen liver sections were cut at 4μm thick, mounted on slides then fixed in PFA 4% at room temperature for 10 minutes. Then slides were washed with PBS (three times) and incubated with 0.2% Triton PBS for 5 minutes at room temperature. After permeabilization sections were incubated
with 75mM NH₄Cl PBS for 30 minutes at room temperature and washed with PBS. Sections were incubated in Blocking Solution I [5% non-fat dry milk, 10% donkey serum, PBS] for 30 minutes at room temperature then washed with PBS. Then slides were incubated in Blocking Solution II [3% BSA, 5% donkey serum, 20mM MgCl₂, 0.3% Triton PBS] for 1 hour at room temperature. Blocking Solution was removed and slides were incubated with rabbit polyclonal antibody to GFP (Table. 1) overnight at 4°C. After three washes with PBS, sections were incubated with AlexaFluor 488 donkey anti-rabbit and DAPI (1/200) for 1 hour at 4°C and mounted with Mowiol. Images were obtained with Confocal Microscope LSM700 (Zeiss). GFP-LC3 puncta were counted (n=3 per group) using ImageJ Software and normalized to the number of DAPI positive cells. For lipid staining, frozen liver sections were cut at 7µm thick on cryostat, mounted on slides then fixed in ice cold 10% formalin for 5-10 minutes. After washings in 60% isopropanol, sections were incubated at room temperature into oil red O dye (Sigma Aldrich) solution for 15 minutes. After incubation, slides were washed in 60% isopropanol for 1 minute then stained in Mayer’s hematoxylin for 30 seconds. Slides were placed in distilled water and mounted with aqueous mounting medium. Images were obtained using x40 objective in Leica DM500 Microscope.30-µm thick brain sections were fixed in PFA 4% in 12-multi-wells plate. For double immunofluorescence analysis, sections were washed with PBS and stained with TUNEL assay kit (Roche) according to manufacturer’s instructions. Then sections were incubated with blocking solution [0.2% Tween, 0.5% FBS, PBS] for 30 minutes at room temperature. The blocking solution was removed and slides were incubated with rabbit polyclonal antibody to beclin-1(BECN1) (Table.1) overnight at 4°C. After three washes with PBS, sections were incubated with AlexaFluor 594 donkey anti-rabbit and DAPI for 1 hour at 4°C. After three washes with PBS, sections were mounted
on glasses with Mowiol. Images were obtained using x63 objective in Confocal Microscope LSM700 (Zeiss). For colocalization analysis sections were incubated with rabbit polyclonal antibody to BECN1, GFAP and, NEUN overnight at 4°C on rotate. After three washes with PBS, sections were incubated with AlexaFluor 488 donkey anti-rabbit, AlexaFluor 594 donkey anti-mouse and, DAPI for 1 hour at 4°C. After three washes with PBS, sections were mounted on glasses with Mowiol. Images were obtained using x63 objective in Confocal Microscope LSM700 (Zeiss).

**Electron microscopy studies.** Liver tissue was fixed with 1% glutaraldehyde prepared in 0.2 M HEPES buffer and post- fixed in 1% OsO4 and 0.5% uranyl acetate. After dehydration through a graded series of ethanol and propylenoxide, the tissue was embedded in the Epoxy resin (Epon 812, Sigma–Aldrich) and polymerized at 60°C for 72 h. EM images of 60 nm thin sections were acquired using a FEI Tecnai-12 electron microscope (FEI, Eindhoven, Netherlands) equipped with a VELETTA CCD digital camera (Soft Imaging Systems GmbH, Munster, Germany). Morphometric analysis of lipid droplets was performed using iTEM software (Olympus SYS, Germany). Number of lipid droplets was counted using the same magnification within 272, 25μm square field of view. These studies have been performed by Lena Polishchuck from the TIGEM Electron Microscopy facility, TIGEM, Pozzuoli, Naples (ITALY).

**Cell studies.** HeLa cells overexpressing, stably, PGRN gene (PG-2) were established by transfecting HeLa cells with pCDNA3 plasmid harboring PGRN gene under the control of the ubiquitous promoter CMV using Lipofectamine 2000 reagent (Thermo Scientific) in
accordance to manufacturer’s procedure. After 48 hours, transfection medium was removed and cells were incubated with selection medium containing G418 Geneticin (0.5mg/mL, Sigma). After 10 days of selection, clones were isolated and grown for 1 week in 10% FBS DMEM (Euroclone). A clone with the highest PGRN expression was selected and grown for further studies. Small interference RNA (siRNA) oligo targeted for human PGRN (pre-designed Rosetta siRNA; Sigma Aldrich) was transfected using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific). A pool of siRNA consisting of scrambled sequences of similar length (Mission siRNA Universal negative Controls, Sigma Aldrich) was transfected as control siRNA. Cells were harvested 48 hours after the transfection. Bafilomycin A1 (Sigma Aldrich) and, SP600125 (Sigma Aldrich) were added to serum-free DMEM culture medium at the dose of 100 nM and, 20mM respectively. HeLa cells were harvested after 2 hours of Bafilomycin treatment and 24 hours post SP600125 treatment.

**RNA extraction and real time PCR.** Total RNA was isolated from tissue and cells with TRIzol (Invitrogen) and RNeasy FFPE Kit (Qiagen) according to manufacturer’s instructions. First-strand cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (AB, Applied Biosystem). PCR reactions were performed with Light Cycler 480 SYBR Green I Master (Roche), Light Cycler 480 (Roche). Primers are listed in Table 2.

**Mouse hippocampal neurons.** Hippocampal neurons were cultured from newborn mice as previously described [87]. Brain regions were dissected in ice-cold Hank’s balanced salt
solution (HBSS), dissociated via trypsinization with 0.05% trypsin-EDTA for 10 min at 37°C, triturated with a siliconized pipette, and plated onto 24-well plastic dishes, coated for 15 min with poly-L-ornithine (Sigma) and after for 30 min with laminin (Sigma). Plating medium MEM (Gibco) supplemented with 5 g l⁻¹ glucose, 0.2 g l⁻¹ NaHCO₃ (Sigma), 0.1 g l⁻¹ transferrin (Calbiochem), 0.25 g l⁻¹ insulin (Sigma), 0.3 g l⁻¹ L-glutamine (Gibco), and 10% horse serum (Gibco) were replaced with MEM containing 5 g l⁻¹ glucose, 0.2 g l⁻¹ NaHCO₃ (Sigma), 0.1 g l⁻¹ transferrin (Calbiochem), 0.3 g l⁻¹ L-glutamine, 2% B-27 supplement (Gibco) and 2 μM cytosinearabinoside (Sigma) 24–48 h after plating. Neurons from wild-type and Grn⁻/⁻ mice were treated for 24 hours with 10mM 3-methyladenine (3-MA), and 12.5mM SP600125 (Sigma). Cells were then incubated with 0.2% trypan blue-HBSS solution (Sigma) for 2 minutes at room temperature and washed with HBSS. Cultured were fixed with phosphate-buffer 4% PFA for 10 minutes at room temperature. Trypan blue positive cells were counted on randomly selected bright fields using x10 objective in Apotome.2 microscope (Zeiss). Lactate dehydrogenase (LDH) activity was measured in cultural medium collected 24 hours after treatment using Lactate Dehydrogenase Assay Kit (BioAssay Systems) in accordance to manufacturer’s procedure. These studies have been performed in collaboration with Irene Sambri from Alessandro Fraldi’s group at TIGEM, Pozzuoli, Naples (ITALY).

**C. elegans studies.** Nematodes were grown and handled following standard procedures, under uncrowded conditions, at 20°C, on NGM (nematode growth medium) agar plates seeded with *Escherichia coli* strain OP50 [88]. Wild-type animals were *C. elegans* variety Bristol, strain N2. The mutant allele used in this work was *pgrn-1(tm985)* kindly provided by C. Kenyon (University of California San Francisco, USA). Well-fed, young adult
animals were used for thrashing assay and backward movement assay to blindly test motor neuron functionality. For thrashing assay, animals were assayed by placing animals in 3 cm diameter plates filled with M9 buffer. The thrashing rate was assessed after 5 minutes, by counting the number of oscillatory bends within a period of 20 seconds and then multiplied by 3 to obtain an estimate of total thrashes per minute. A single thrash was defined as a complete change in the direction of the body down the midline. Each bar represents the mean of two independent experiments conducted for both strains. The statistical significance was determined using the Mann-Whitney test comparing pgrn-1(tm985) against wild-type strain. The backward movement assay was performed blind on NGM plates, 6 cm in diameter, seeded with bacteria. Using an eyelash the animal was touched first on the tail to induce a forward movement and then on the head to test the backward movement. Defective backward locomotion was scored when animals were unable to fully move backward after a mechanical stimulus on the hand. For each data set the percentage of animal with normal backward movement among the total number of tested animals was calculated. Each bar represents the mean of two independent experiments conducted for both strains. The statistical significance was determined using z statistic test and comparing pgrn-1(tm985) mutant against the wild-type strain. Drug assays were performed according to published procedures [89, 90]. Drugs were purchased from Sigma. In the aldicarb test reflecting cholinergic neuron function[91], young adult hermaphrodites were placed on plates containing aldicarb at a concentration of 1 mM and the effects on animal movement were scored at intervals of 15 minutes, until two hours. Animals were scored as paralyzed when no body movements were observed after a mechanical stimulus. For the 3-MA assay, the animals were synchronized by egg extraction using sodium hypochloride and incubated at 20°C for 24h on unseeded agar
plates. Animals were then treated with 3-MA dissolved in water at a concentration of 10 mM, and incubated in liquid medium (M9 buffer plus cholesterol), supplemented with bacteria, for 24h. Then animals were recovered from the medium and transferred on seeded agar plates until they reached the young adult stage. Animals were then randomly picked and tested in the thrashing assay. These studies have been performed in collaboration with Ivan Gallotta from Elia Di Schiavi’s group at CNR, Naples (ITALY).

**Statistical analyses.** For body weight and glucose tolerance tests, data were analyzed by time series (Conesa A and Nueda MJ. maSigPro, Significant Gene Expression Profile Differences in Time Course Microarray Data. R package version 1.38.0). For GFP-LC3 and trypan blue staining, data expressed as average ± SEM and were analyzed by General Linear Model (GLM) test. The remaining comparisons were made by Student’s t-test. Statistical analyses were performed by Annamaria Carissimo, Bioinformatic Core, TIGEM.

**Table 1.** Primary antibodies for immunoblot and staining.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species in which the Ab was raised</th>
<th>Source</th>
<th>Code</th>
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</thead>
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</tr>
<tr>
<td>Gene</td>
<td>Species</td>
<td>Sequence (5’→3’)</td>
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**Table 2.** Primers for real time PCR.
Results

**PGRN overexpression results in obesity, lipid accumulation, and reduced glucose tolerance**

To investigate the role of PGRN overexpression *in vivo*, I delivered the *PGRN* gene to the liver of 8 week-old wild-type C57B6/L mice by an intravenous injection of helper-dependent adenoviral (HDAd) vector (HDAd-PGRN). Control mice were injected with the same dose of an HDAd-AFP vector expressing the unrelated, non-toxic, non-immunogenic α-fetoprotein (AFP) reporter gene under the control of the same expression cassette of the HDAd-PGRN vector [92]. Consistent with previous studies [84, 93, 94], transgene expressions (AFP and PGRN) were sustained long-term for the entire period of observation (12 months post-injection for mice under regular diet and 6 months in the mice fed with high fat diet; Fig. 1A-B). Mice overexpressing PGRN showed greater increase in body weight compared to controls (Fig. 2A). The weight gain was not induced by changes in feeding behavior, as shown by the food intake that was not different between mice injected with HDAd-PGRN or HDAd-AFP (Fig. 2B). Livers of mice sacrificed at 12 months post-vector injections had no differences in weight (Fig. 2C) but an increased weight of epididymal fat was detected in mice injected with HDAd-PGRN compared to HDAd-AFP injected controls (Fig. 2D). Compared to controls, mice overexpressing PGRN showed decreased glucose tolerance (Fig. 3A) elevated fasting serum cholesterol and triglycerides, and reduced serum free glycerol (Fig. 3C). Oil red staining of livers revealed increased lipid accumulation in HDAd-PGRN injected mice compared to controls (Fig. 3D). Ultrastructural analysis of liver sections showed an increased number of lipid droplets in PGRN overexpressing mice compared to control mice (Fig. 3E). Consistent with the results in HeLa cells [36], an increase in lysosomal size was also observed in livers of mice
overexpressing PGRN (Fig. 3F). A similar increase in body weight but at earlier time points was observed in PGRN overexpressing mice fed with high fat diet (Fig. 2A). These mice also exhibited a significant increase in epididymal fat weight compared to HDAd-AFP injected controls (Fig. 2D) but did not show differences in food intake and liver weight (Fig. 2B, C). Impaired glucose tolerance was also observed in high fat diet mice (Fig. 3B). Taken together, these results show that hepatic overexpression of PGRN results in increased body weight and fat content, altered blood and liver lipid contents, and impaired glucose tolerance. This phenotype occurs earlier in mice under HFD compared to mice fed with a regular diet.

Figure 1. (A-B) Serum AFP and PGRN levels in wild type mice injected with HDAd-AFP (n=4) or HDAd-PGRN (n=4) fed with regular diet (blue lines) or high fat diet (red lines).
Figure 2. (A) Body weight curves in wild-type mice injected with HDAd-PGRN (n=9; closed circles) or HDAd-AFP (n=8; open squares) fed with a regular diet (blue lines) or high fat diet (red lines) (time series analysis, p<0.05). (B) Food intake in wild type mice injected with HDAd-AFP (n=8; open bar) and HDAd-PGRN (n=10; closed bar) fed with regular diet (blue bar) or high fat diet (red bar) (HDAd-AFP, n=4 HDAd-PGRN, n=4). (C) Weight of livers of HDAd-PGRN (n=9; closed bar) or HDAd-AFP (n=9; open bar) injected mice fed with regular diet (blue bar) or high fat diet (red bar). (D) Weights of epididymal fat in HDAd-PGRN (n=13; blue bar) or HDAd-AFP (n=11; open bar) injected mice fed with regular diet (blue bar) or high fat diet (red bar). *: p<0.05.
Figure 3. (A) Glucose tolerance test in wild-type mice injected with HDAd-PGRN (n=14; closed circles) or HDAd-AFP (n=12; open squares) fed with regular diet following intraperitoneal glucose injection (time series analysis, p<0.05). (B) Glucose tolerance test in wild-type mice injected with HDAd-PGRN (n=14; closed circles) or HDAd-AFP (n=12; open squares) fed with high fat diet following intraperitoneal glucose injection (time series analysis, p<0.05). (C) Serum cholesterol, triglycerides, and glycerol in wild-type mice injected with HDAd-PGRN (PGRN; n=4, blue bars) or HDAd-AFP (AFP; n=4, open bars). (D) Representative images of oil red staining of wild-type mice injected with HDAd-PGRN (PGRN) or HDAd-AFP (AFP). (E) Lipid droplets visualized by electron microscopy and quantitation of wild-type mice injected with HDAd-PGRN (PGRN; n=3 per group, blue bars) or HDAd-AFP (AFP; n=3 open bars). (F) Lysosomal diameter by electron microscopy and quantitation of wild-type mice injected with HDAd-PGRN (PGRN; n=3 per group, blue bars) or HDAd-AFP (AFP; n=3 open bars). *: p<0.05.
PGRN overexpression results in reduced IGF-1 and impaired autophagy in vivo.

A variety of adipokines play pivotal roles in energy homeostasis by affecting insulin sensitivity, glucose and lipid metabolism, food intake, and inflammation [95]. To investigate the phenotype of PGRN overexpressing mice, I analyzed the circulating adipokine profile of mice injected with HDAd-PGRN. Several adipokines were differently expressed in PGRN overexpressing mice compared to controls (Fig. 4A). IGF-1 was among the most decreased adipokines (Fig. 4A). I focused on IGF-1 because of its recognized role both in metabolic syndrome and neurodegeneration [96]. In PGRN overexpressing mice, IGF-1 (both protein and mRNA) reduction was confirmed in both sera and livers (Fig. 4B-C). Because of the growing evidence supporting the role of autophagy in lipid accumulation [97] and the role of IGF-1 in autophagy and metabolic syndrome [96, 98], I hypothesized that autophagy is impaired under conditions of PGRN overexpression resulting in accumulation of hepatic lipids, obesity, and reduced glucose tolerance. The autophagy impairment was confirmed in vivo in mice overexpressing PGRN that showed increased hepatic levels of LC3 and p62 (Fig. 4D) and in GFP-LC3 transgenic mice injected with HDAd-PGRN showing increased LC3 positive puncta (Fig. 4E).
Figure 4. (A) Profile of serum adipokines expressed as fold difference of HDAd-PGRN injected (n=2) compared to HDAd-AFP injected mice (n=2). *: p<0.05. (B-C) Serum IGF-1 and liver mRNA IGF-1 in HDAd-PGRN (PGRN; n=4 per group, blue bars) and HDAd-AFP injected mice (AFP; n=4 open bars). (D) Western blotting for LC3 and p62 in HDAd-PGRN and HDAd-AFP injected wild-type mice (each lane corresponds to a single mouse); β-actin was used as loading control. (E) Representative images of immunofluorescence for LC3 puncta in livers of GFP-LC3 mice injected with HDAd-PGRN (PGRN) or HDAd-AFP (AFP) and quantification (n=3 per group).
Loss of PGRN results in JNK activation and increased IGF-1 expression leading to excessive autophagy in brain

PGRN binds and antagonizes TNFR [19]. Stimulation of TNFR leads to the activation of JNK by phosphorylation. JNK phosphorylates BCL2 that then dissociates from BECN1 leading to the formation of the BECN1-related PI3K III complex which promotes autophagy [99]. Moreover, activated JNK phosphorylates and increases BECN1 transcription [100]. Given the impaired autophagy in livers overexpressing PGRN, I hypothesized that loss of PGRN would result in the opposite effect on autophagy (i.e. excessive activation) through JNK activation due to TNFR over-stimulation. First, I found that HeLa cells stably over-expressing PGRN (HeLa-PG2; Fig. 5A-B) showed increased LC3-II and p62, and decreased BECN-1 (Fig. 5C, D). Incubation of HeLa-PG2 cells with the lysosomal inhibitor bafilomycin A1 did not increase LC3-II and p62 consistent with a block in autophagy flux (Fig. 5E). In contrast cells knocked-down for PGRN (Fig. 5A, B) showed increased LC3-II and BECN1, and reduced p62 (Fig. 6A-B; Fig. 9A-C), suggesting increased autophagy. Following incubation with bafilomycin, a further increase of LC3-II levels and higher amounts of p62 were detected in PGRN knock-down cells, thus confirming an increase of autophagy flux due to PGRN loss (Fig. 6A; Fig. 9A, C). Moreover, PGRN knock-down cells showed an increase of JNK phosphorylation (Fig. 6C) while the increase in LC3-II and the reduction in p62 were abrogated by JNK inhibitor SP610025, suggesting that autophagy activation occurs via JNK (Fig. 6C; Fig. 9D, E). Next, I found that autophagy was increased in brains of Grn-/- mice, as shown by increased levels of LC3-II and BECN1 (both mRNA and protein), and reduced p62 (Fig. 7A-C; Fig. 10A-C). Compared to controls, brains of 12-week-oldGrn-/- mice showed increased JNK and BCL-2 phosphorylation (Fig. 7D, E; Fig. 10D, E). Next I evaluated IGF-1 levels in PGRN knock- down cells and Grn-/- brains. IGF-1 level was decreased in
PGRN overexpressing cells but was increased in PGRN knock-down cells (Fig. 8A). Moreover, IGF-1 levels (both mRNA and protein) were increased in brains and sera of Grn/- mice compared to controls (Fig. 8B-D). Autophagy can be induced in neurons by an MTOR independent pathway involving IRS-2 activation [101]. IRS-2 tyrosine phosphorylation was indeed increased in Grn/- brains (Fig. 8E; Fig. 10F). Taken together, these results showed IGF-1 upregulation and increased IRS-2 activation in Grn/- mouse brains that likely contributes to the enhancement of autophagy. Importantly, brain sections of Grn/- brains showed marked increased staining of TUNEL and BECN1 that co-localized (Fig. 11A) suggesting that cell death is dependent on increased autophagy in brain. Interestingly, BECN1 signal was found in NeuN-positive cells of the brain (Fig. 12A), was partially detected in IBA-1-positive microglia cells (Fig. 12A) but was not detected in GFAP-positive astrocytes (Fig. 12A), suggesting that increased activation of autophagy mostly occurred in neuronal cells.
**Figure 5.** (A-B) Real time PCR and Western blotting for PGRN in un-transfected wild-type HeLa cells (WT), in HeLa cells stably overexpressing PGRN (PG-2), in HeLa cells knocked-down for Grn (PGRN KD), and in HeLa cells transfected with scramble siRNA (siRNA scr). β-actin was used as loading control. (C-D) LC3, p62, and BECN1 in un-transfected wild-type cells (WT) and in cells stably overexpressing PGRN (PG-2). (E) Incubation of HeLa-PG2 cells with bafilomycin (Bafilo) did not increase LC3-II or p62 protein levels. GAPDH was used as loading control.
Figure 6. (A) LC3 and p62 in cells knocked-down for PGRN (PGRN KD), and in cells transfected with scramble siRNA (siRNA scr) untreated and incubated with bafilomyicin (Bafilo). β–actin was used as loading control. (B) BECN-1 in cells knocked-down for PGRN (PGRN KD), and in cells transfected with scramble siRNA (siRNA scr). β–actin was used as loading control. (C) Phospho-JNK, total JNK, LC3 and p62 in cells knocked-down for PGRN (PGRN KD), and in cells transfected with scramble siRNA (siRNA scr) incubated with SP600125 or vehicle alone. β–actin was used as loading control.
Figure 7. (A,B) Western blotting for LC3, p62 and, BECN1 in brains of Pgrn-/- or age and gender-matched control (WT) mice (each lane corresponds to an individual mouse). β–actin and GAPDH were used as loading controls. (C) Real time PCR for BECN1 in brains of pgrn-/- or age and gender-matched control (WT) mice (n=4 per group). (D) Phospho-JNK and total JNK in brains of pgrn-/- or age and gender-matched control (WT) mice (each lane corresponds to an individual mouse). (E) Phospho-Bcl-2 (at Ser70 residue) and total Bcl-2 in brains of pgrn-/- or age and gender-matched control (WT) mice (each lane corresponds to an individual mouse). β–actin was used as loading control.
Figure 8. (A) Real time PCR for IGF-1 in un-transfected wild-type HeLa cells (WT), in HeLa cells stably overexpressing PGRN (PG-2), in HeLa cells knocked-down for PGRN (PGRN KD), and in HeLa cells transfected with scramble siRNA (siRNA scr). (B) Real time PCR for IGF-1 in brains of Grn−/− or age and gender-matched control (WT) mice (n=4 per group). (C-D) Serum and brain IGF-1 levels in Grn−/− and in age and gender-matched control wild type (WT) mice. (E) Western blotting for tyrosine phosphorylation after immunoprecipitation of brain lysates with anti-IRS-2 antibody (each lane corresponds to an individual mouse). *: p<0.05.
Figure 9. Quantitations of band densities of western blottings performed as duplicates and shown in Fig. 6 for LC3-II (A, D; Fig. 6A and 6C, respectively), BECN1 (B; Fig. 6B), or p62 (C, E; Fig. 6A and 6C, respectively) normalized to β-actin in cells knocked down for PGRN (PGRN KD) or transfected with siRNA scramble (siRNA scr) untreated, incubated with bafilomycin or SP610025. Averages ± SD are shown; *: p<0.05.
Figure 10. Quantitations of band densities of western blottings shown in Fig. 7 for LC3-II (A; Fig. 7A), BECN1 (B; Fig. 7B), p62 (C; Fig. 7B), normalized to β-actin or GAPDH. Quantitations of band densities of western blottings shown in Fig. 7 for phospho-JNK normalized to total JNK (D; Fig. 7D), phospho-BCL-2 normalized to total BCL-2 (E; Fig. 7E), and phospho-IRS-2 normalized to total IRS-2 (F; Fig. 8E). At least n=3 per group. Averages ± SD are shown; *: p<0.05.
Figure 11. (A) Confocal analysis of BECN1 and TUNEL staining in brains of WT and Grn−/− mice (3 per group).
Figure 12. (A) Colocalization analysis of BECN1 with GFAP (upper panel), NEUN (middle panel) and IBA1 (lower panel) in brains of Grn-/- mice (3 per group).
**Neuronal loss and dysfunction by PGRN loss is rescued by autophagy inhibition**

To investigate the role of autophagy in the pathogenesis of FTLD, I evaluated viability of hippocampal neurons isolated from brains of Grn−/− mice or WT controls in the presence of the standard autophagy inhibitor 3-methyladenine (3-MA) suppressing the activity of Vps34, a class III PI3K that interacts with BECN1 in vesicle nucleation during autophagy induction [102, 103] or the JNK inhibitor SP600125. In sharp contrast to WT neurons which showed increased cell death by Trypan Blue staining and release of lactate dehydrogenase (LDH) in culture medium, Grn−/− neurons exhibited reduced mortality following 3-MA or SP600125 incubation (Fig. 13A-F).

Next, I used pgrn-1(tm985) null *C. elegans* [104, 105] to investigate autophagy in pgrn-deficient neurons. *pgrn-1* mutants have unaltered apoptotic initiation but increased clearance of apoptotic cells [104]. Moreover, they are more resistant to several stressors, such as osmotic or heat shock stress [104]. I evaluated both GABAergic and cholinergic motor neurons that regulate locomotion in *C. elegans*. A significantly reduction of thrashing rate reflecting cholinergic neuron function [91] was detected in *pgrn-1 (tm985)* mutants (Fig. 14A). Moreover, *pgrn-1(tm985)* mutants were more resistant to aldicarb-induced paralysis [106] compared to wild-type animals (Fig. 14B). Blocking of autophagy by 3-MA rescued the thrashing defect and restored resistance to aldicarb-induced paralysis of *pgrn-1(tm985)* mutants (Fig. 14A, B).
Figure 13. (A) Trypan blue staining of hippocampal neurons isolated from Grn-/ and wild-type control (WT) mice at post-natal day 2 treated with 3-methyladenine (3-MA) or vehicle (n=3 mice per group). (B) Quantification of trypan blue-positive neurons per field of view (at least n=5 fields analyzed). Averages ± SD are shown. (C) LDH in media of hippocampal neurons from Grn-/ and wild-type control (WT) mice treated with 3-MA or vehicle (n=3 mice per group). Averages ± SD are shown. *: p<0.05. (D) Trypan blue staining of hippocampal neurons isolated from Grn-/ and wild-type control (WT) mice at post-natal day 2 treated with SP600125 or vehicle (n=3 mice per group). (E) Quantification of trypan blue-positive neurons per field of view (at least n=5 fields analysed). Averages ± SD are shown. *: p<0.05. (F) LDH in media of hippocampal neurons from Grn-/ and wild-type control (WT) mice treated with SP600125 or vehicle (n=3 mice per group). Averages ± SD are shown. *: p<0.05.
Figure 14. (A) *pgrn-1(tm985)* null mutant *C. elegans* have a defect in thrashing, a behavior mediated by acetylcholine motor neurons. Each bar represents the mean of the thrashing rate per minute± SEM. The controls are wild-type animals (WT). Number of animals tested for control n=15 and for *pgrn-1(tm985)* n=30. 3-MA treatment rescues the thrashing phenotype of *pgrn-1(tm985)* mutants. Number of animal tested, from left to right, 27-21-18-21. *: p<0.004 (Mann-Whitney test). (B) *pgrn-1(tm985)* mutants are more resistant to aldicarb, a reversible acetylcholinesterase inhibitor that results in increased acetylcholine at the neuromuscular junction leading to hyperactivation of acetylcholine receptors, muscle hypercontraction, and paralysis. Therefore a time-course of the aldicarb-induced paralysis provides a simple read-out for the analysis of cholinergic transmission at the neuromuscular junction. A strain resistant to aldicarb-induced paralysis has impairment in the cholinergic transmission, hence the resistancy to aldicarb induced paralysis which can be due to a reduced release (or synthesis) of acetylcholine in the synaptic cleft. After one hour of aldicarb treatment, only 40% of *pgrn-1* null mutants are paralyzed compared to 100% for the wild-type strain (WT). Number of animal tested for wild-type strain n=20 and for *pgrn-1(tm985)* n=63. 3-MA rescued the defect in *pgrn-1* null mutants (p<0.001; Mantel-Cox test). Averages ± SD are shown; *: p<0.05.
Discussion

Role of PGRN overexpression in vivo and its effects on liver autophagy

PGRN has recently emerged as an important regulatory protein of glucose metabolism and insulin sensitivity. Interestingly, PGRN plasma levels positively correlate with insulin resistance, the hallmark of type 2 diabetes. To investigate the link between PGRN and metabolism I delivered GRN gene in the liver of WT mice by the injection of HDAd vector harboring GRN cDNA under the control of liver-specific promoter thus obtaining a strong and sustained long-term liver-specific expression of the transgene for the entire period of observation. I found that PGRN overexpression in livers results in obesity, hepatic lipid accumulation, and reduced glucose tolerance in mice consuming regular diet. This phenotype appears earlier in PGRN-injected mice consuming HFD. The involvement of PGRN in glucose and insulin sensitivity has been first identified in adipocytes that developed insulin resistance after the treatment with recombinant PGRN protein. In a more recent study it has been demonstrated that short-term treatment with recombinant PGRN protein induces impaired glucose ad insulin metabolism in WT mice whereas Grn/- mice are protected from HFD-induced insulin resistance [23]. However, in contrast to my work, that study failed to show increase in body weight, hepatic lipid droplets and, triglycerides following 21 days of injections of PGRN recombinant protein [23]. This difference may be explained by the reduced time (21 days) mice were injected with PGRN compared to liver-specific long term (48 weeks) expression at high levels achieved in my study.

I previously demonstrated that PGRN overexpression induces an increase of the levels of lysosomal proteins and, lysosomal size in human cells [36]. Interestingly, I
observed that PGRN overexpressed livers also show an increased lysosomal size assessed by electron microscopy.

Lysosomal function is strongly linked to autophagy pathway. In the present study, I found that PGRN overexpression is associated also to an impairment of autophagy both in vitro and in livers.

Autophagy transpires at low basal levels in all cells to serve many homeostatic functions. Recent studies have shown that autophagy plays an important role in lipid homeostasis [48]. The pharmacological and genetically inhibition of autophagy significantly increased hepatocyte lipid content thus indicating that autophagy is required for lipid breakdown and turnover [48]. In my work, I found that PGRN overexpression leads to impaired autophagy and this may cause the hepatic lipid accumulation and the obesity phenotype observed in PGRN overexpressed mice.

Moreover, I found that PGRN gain of function induces a reduction of the transcriptional levels of IGF-1, an adipokine mainly involved in metabolism. Infact, dysregulated insulin/IGF-1 signaling can contribute to the development of metabolic diseases such as type 2 diabetes [107].

The link between PGRN and IGF-1 signaling has been firstly demonstrated in adipocytes that showed inhibited insulin-induced activation of IRS-1 and AKT after treatment with recombinant PGRN. However, how PGRN affects IGF-1 expression at transcriptional level remains to be elucidated.
Role of autophagy in PGRN deficient brains

PGRN is one in a growing list of proteins with roles in both metabolic disease and neurodegenerative disorders. Loss-of-function mutations in GRN gene represent the major genetic cause of FTLD, the most common cause of early-onset neurodegenerative dementia in cases before 65 years of age [25]. Importantly, little is known about the molecular mechanisms underlying neurodegeneration due to PGRN deficiency. In the present work, I found that PGRN loss of function is associated to autophagy activation as demonstrated by the increase of autophagic proteins LC3 and BECN1 and decrease of autophagy receptor p62 in PGRN deficient cells and brains. Autophagy induction is generally viewed as neuroprotective [108] but has also been implicated as a mechanism of programmed cell death [54]. Autophagy cell death is characterized by an increase in autophagosomes synthesis and flux that leads to the degradation of vital component of the cells [54]. Interestingly, I found that increased autophagy is associated with neurodegeneration induced by PGRN and inhibition of autophagy had beneficial effects on neurodegeneration induced by PGRN loss of function. Consistent with the increased brain autophagy, I found that the autphagic protein BECN1 co-localizes with TUNEL in Grn-/- brain, and BECN1 largely co-localized with the neuronal marker NeuN, suggesting that activation of autophagy and cell death mostly occurred in neurons.

To show the beneficial effect of autophagy inhibition, I used both hippocampal neurons from Grn-/- mice and the pgrn-1(tm985) C. elegans null mutant [104]. Importantly, treating Grn-/- neurons with the standard autophagy inhibitor 3-MA ameliorates cell viability assessed by Trypan Blue staining and LDH activity. Moreover, I found that PGRN deficient C. elegans show a locomotor defect phenotype that is rescued by treatment with 3-MA. Despite the generally neuroprotective role of autophagy, my
findings suggest that activation of autophagy is detrimental in *Grn/-* brains and may contribute to neurodegeneration induced by PGRN loss.

Once assessed the activation of autophagy in *Grn/-* mouse brains, I next investigated the mechanism underlying PGRN loss-of-function induced autophagy and I focused my attention on TNF-α/JNK signaling. PGRN binds TNFR with an antagonistic effect [19]. JNK is potentially activated by TNF-α and once activated phosphorylates BCL2 disrupting the interaction between BECN1-BCL2 thus inducing autophagy [76]. Interestingly, I found a marked activation of JNK and phosphorylated BCL2 associated to increased levels of BECN1 in both PGRN deficient cells and brains. The inhibition of JNK rescued the increase of the autophagic marker, LC3-II, in PGRN knocked-down cells suggesting that the activation of autophagy occurs via JNK signaling. Importantly, I found that the treatment with SP600125, a specific inhibitor of JNK, improves the viability of *Grn -/-* neurons. Activation of JNK signaling has been strongly associated to ACD in different neurodegenerative diseases. In particular, administration of SP600125 results in reduced cell death associated to decreased autophagy in a mouse model of AD [81] that share many common features with FTLD. Taken together, my results show that PGRN deficiency induces autophagy that seems to play a cytotoxic role in brain. Activation of autophagy occurs via JNK signaling that is activated due to TNFR over-stimulation.

Moreover, in my work I found that PGRN loss-of-function induces a significantly increase in the transcriptional levels of IGF-1 and in the levels of activated IRS-2. Several studies have shown that reduction of IGF-1/IRS-2 signaling pathway plays a protective role in neurodegeneration. Infact, it has been shown that AD mice lacking *Irs*-2 gene show a marked reduction of Aβ plaque in the brain and an improvement in learning and memory [109]. Huntington disease mice show increased level of IRS-2 that accelerates the
progression of the neurodegeneration and reduced the lifespan [110]. In addition, reduction of IRS-2 is associated to accumulation of fewer polyQ-HTT in the brain [110]. Interestingly, deficiency of insulin/IGF-1 signaling attenuates neurotoxicity and aggregation of TDP-43 protein, a hallmark of FTLD pathology [111]. Moreover, IGF-1 pathway can negatively regulate autophagy by activating MTOR, a well known inhibitor of autophagy in nutrients condition [112]. Additionally, IGF-1 can induce autophagy in an MTOR independent manner via IRS-2 activation [101]. Phosphorylation of IRS-2 leads to the activation of PI3K III that in turn forms a multiprotein complex including BECN1 required to autophagy initiation [101]. Importantly, although in most tissues insulin and IGF-1 signal via IRS-1 and IRS-2 [113], in brain they signal predominantly via IRS-2 [114]. Grn/- brains show a marked increase in IRS-2 phosphorylation together with an increase in BECN1, suggesting that IGF-1 signaling pathway might exacerbate autophagy activation induced by JNK in Grn/- mouse brains via IRS-2 activation.

Conclusions and future perspectives

In conclusion, in my thesis work, I demonstrated that gain and loss of function of PGRN have opposite effect on autophagy. Hepatic PGRN overexpression is associated to impaired autophagy leading to metabolic syndrome whereas PGRN deficiency leads to increased autophagy in brains. Although autophagy induction is generally viewed as neuroprotective, I found that increased autophagy is associated with neurodegeneration induced by PGRN loss and its inhibition ameliorates neuronal viability and dysfunction.

I also found that JNK signaling is involved in the pathogenesis of FTLD by inducing autophagy and its inhibition seems to play a neuroprotective role. Additionally,
activation of IGF-1/IRS-2 pathway may contribute to enhance JNK-induced autophagy leading to neurodegeneration in Grn null mouse brain (Figure 15).

FTLD is the most common early-onset type of dementia after Alzheimer disease and there is no current available therapy. By establishing molecular links among the TNFR signaling, IGF-1, JNK and autophagy, my thesis work unraveled novel mechanisms involved in neurodegeneration induced by PGRN loss and open new avenues for therapeutic studies in FTLD.

However, the link between autophagy and neurodegeneration due to PGRN deficiency in vivo remains elusive. Based on the beneficial effect of 3-MA on worms and in mouse neurons deficient for PGRN incubated ex vivo with 3-MA and SP600125, I will administer either the autophagy inhibitor 3-MA or the JNK inhibitor SP600125 to PGRN deficient mice and following treatments I will evaluate the brains for signs of neurodegeneration. In addition, I will test the levels of autophagy markers and phosphorylated JNK in whole brain extracts of injected mice that I expect to be reduced compared to controls mice.

Based on the results of my thesis work, I expect that treatments of PGRN deficient mice with the autophagy and JNK inhibitors will result in improved neurologic phenotype. Importantly, both drugs have been found to cross the blood-brain-barrier and can be injected to mice without harmful effects [115]. In vivo inhibition of autophagy by 3-MA attenuates traumatic brain injury-induced cell death and behavioral deficits [116]. Moreover, SP600125 treatment has been found to have beneficial effect on neurophatology of AD mice [115]. Therefore, I expect that inhibition of autophagy will improve neuropathology in PGRN deficient mice.
In the present study, I showed that PGRN deficiency leads to increased brain levels of IGF-1 and to the activation of its signaling pathway. However, the role of IGF-1 in autophagy induced cell death in FTLD needs to be further investigated. To do that, I will administer specific inhibitors of IGF-1 to both PGRN deficient neurons and mice and I will evaluate cell viability and signs of neurodegeneration in the brain as well as the levels of autophagy proteins. Inhibition of IGF-1 signaling pathway has been found to be neuroprotective in several neurodegenerative diseases [109]. Therefore, I expect that inhibition of IGF-1 pathway will improve neuropathology in PGRN deficient mice. Taken together, my future planes could allow me to consider autophagy, JNK and IGF-1 inhibitors, as potential therapeutic agents for FTLD.
**Figure 15.** Proposed model for neurodegeneration in Grn−/− brains. PGRN loss of function induces activation of autophagy via JNK due to TNFR over-stimulation. PGRN loss also induces an increase of IGF-1 level that activates autophagy via IRS-2 activation. Excessive autophagy leads to neurodegeneration.
References


