Revised Ms. # NBA-13-58

PGRN haploinsufficiency increased Wnt5a signaling in peripheral cells from FTL D-progranulin mutation carriers

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Running header: non-canonical Wnt5a signaling is enhanced in FTL D-TDP cells.

Key words: FTL D-TDP, lymphocytes, cell proliferation, CDK6, Wnt5a signaling. Pertussis toxin

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ABSTRACT

Loss-of-function progranulin (PGRN) mutations have been identified as the major cause of frontotemporal lobar degeneration with TDP-43 protein inclusions (FTLD-TDP). Previously, we reported cell cycle-related alterations in lymphoblasts from FTLD-TDP patients, carrying the c.709-1G>A null PGRN mutation, suggesting aberrant cell cycle activation in affected neurons. Here we report that PGRN haploinsufficiency activates the ERK1/2 pathway in a Ca^{2+} , protein kinase C-dependent and pertussis toxin-sensitive manner. Addition of exogenous PGRN or conditioned medium from control cells normalized the response of PGRN deficient lymphoblasts to serum activation. Our data indicated that non-canonical Wnt5a signaling might be overactivated by PGRN deficiency. We detected increased cellular and secreted levels of Wnt5a in PGRN deficient lymphoblasts associated with enhanced phosphorylated calmodulin Kinase II (pCaMKII). On the other hand, treatment of control cells with exogenous Wnt5a activated CaMKII, increased ERK1/2 activity and cell proliferation up to the levels found in c.709-1G>A carrier cells. PGRN knockdown SH-SY5Y neuroblastoma cells also show enhanced Wnt5a content and signaling. Taken together, our results unveiled an important role of Wnt signaling in FTLD-TDP pathology and suggest a novel target for therapeutic intervention.

1. INTRODUCTION

Frontotemporal dementia (FTD) is the second most common cause of presenile dementia (Ratnavalli, et al., 2002). At present, several genes have been associated with the FTLD pathology, including microtubule associated protein tau gene (MAPT), progranulin (GRN), valosin-containing protein (VCP)-1, chromatin-modifying 2B (CHMP2B) (Baker, et al., 2006, Cruts, et al., 2006, Holm, et al., 2007, Rademakers, et al., 2004, Watts, et al., 2004) and, very recently, the novel C9ORF72 hexanucleotide expansion in chromosome 9 responsible for familial FTD-MND cases and amyotrophic lateral sclerosis (ALS) (DeJesus-Hernandez, et al., 2011, Renton, et al., 2011). The pathology of FTD associated with PGRN mutations is characterized by ubiquitin positive TDP-43 inclusions and absence of tau pathology, and thus named FTD-TDP (Eriksen and Mackenzie, 2008, Neumann, et al., 2006, Premi, et al., 2012). PGRN mutations are dominantly inherited and the disease mechanism is postulated to be haploinsufficiency, as most PGRN mutations lead to an approximately 50% reduction in PGRN levels (Ahmed, et al., 2007, Coppola, et al., 2008, Cruts and Van Broeckhoven, 2008).

PGRN was first identified as a gene that was overexpressed in epithelial tumors and it was further found to be a player in wound healing and inflammation (Zhu, et al., 2002). The full-length human PGRN transcript encodes the PGRN precursor of 593 amino acids with a predicted molecular mass of 63.5 KDa. PGRN is secreted and cleaved by extracellular proteases like elastase, producing mature granulins (Zhu, et al., 2002). Progranulin and granulins have been known to function as growth factors, exerting opposing effects on cell growth and neurite outgrowth (Van Damme, et al., 2008). Nevertheless, the function of PGRN in the central nervous system (CNS) is poorly understood and it has only been sparsely studied (Neumann, et al., 2009, Rohrer, et al.,

2009). Therefore, unraveling the function of PGRN in the CNS would be of great value in the development of disease-modifying therapies.

It is becoming well recognized the involvement of cell cycle-related events in neurodegenerative disorders (Herrup and Yang, 2007, Hoglinger, et al., 2007, Ueberham and Arendt, 2005). Postmitotic neurons in the affected regions show aberrant induction of cell cycle regulators, which is believed to induce detrimental consequences for neurons (Bonda, et al., 2010, Lee, et al., 2011). Regarding FTLD, the presence of mitotic markers in the frontal cortex of 2 individuals diagnosed FTLD-17 from the Seattle BK family carrying the Val279Met tau-mutation was previously reported (Husseman, et al., 2000). These authors also reported changes in cell cycle regulatory proteins in other FTLD-tau diseases such as corticobasal degeneration (CBD) or Pick disease. More recently, the presence of phosphorylated retinoblastoma protein (pRb) has been detected in the brain of patients suffering from a number of taupathies including FTD (Stone, et al., 2011). On the other hand, a possible link between FTLD-TDP and altered cell cycle control was reported (Ayala, et al., 2008). These authors described a loss of functional nuclear TDP-43 in cultured PGRN deficient neurons, thereby decreasing its inhibitory transcriptional regulation of CDK6, which plays a key role in G₁ progression (Weinberg, 1995).

Previous work in our laboratory tried to unravel the molecular consequences of PGRN deficiency in the proliferative response of immortalized lymphocytes from carriers of a prevalent ancestral loss-of-function PGRN mutation related to Basque population (c.709-1G>A) (Lopez de Munain, et al., 2008, Moreno, et al., 2009). Enhanced proliferation of PGRN deficient cells, associated with upregulation of CDK6, was shown when compared with control cells (Alquezar, et al., 2011). Thus, it seems that in FTLD-TDP, as is the case for other neurodegenerative disorders, peripheral cells from

patients may show a disease-specific signature (de las Cuevas, et al., 2003, Stieler, et al., 2012). In this work we use lymphoblastoid cell lines obtained by infecting peripheral blood mononuclear cells with the Epstein Barr virus (EBV). Previously, we demonstrated that EBV-immortalized lymphocytes retain the cellular response of fresh obtained lymphocytes, to serum addition or withdrawal, finding similar changes in cell proliferation and survival, as well as in the content of certain regulatory proteins to those found in untransformed lymphocytes (Bartolome, et al., 2007, Munoz, et al., 2008). Therefore, easily accessible blood cells from FTLD-TDP patients could be a useful model to study cell cycle-related events in FTLD-TDP pathology.

In this work, we have analyzed potential alterations in signaling pathways induced by PGRN haploinsufficiency to explain the altered cellular response to serum stimulation. It is known that PGRN interacts, outside and inside the cell, with signaling transduction pathways to regulate a wide number of cellular functions. For example, PGRN can activate various growth factor signaling pathways involved in cell growth/survival, including extracellular regulated kinase (ERK), phosphatidyl inositol-3 kinase (PI3K)/Akt and p70S6 (He, et al., 2002, Monami, et al., 2006, Zanocco-Marani, et al., 1999). More recently, it has been reported that PGRN may be involved in Wnt signaling (Rosen, et al., 2011). To address the influence of PGRN deficit in cell signaling, we use specific inhibitors of main pathways as well as conditioned medium from control and PGRN deficient lymphoblasts or exogenous recombinant human PGRN (rhPGRN). Our results show that the specific signaling cascade implicated in the upregulation of CDK6 activity and cell proliferation of PGRN mutation bearing lymphoblasts is Ca^{2+} , Protein Kinase C (PKC), and pertussis toxin (PTX)-dependent and involves activation of the ERK1/2 pathway. Evidence is presented that non-canonical Wnt5a may be overactivated in PGRN deficient cells.

2. MATERIALS AND METHODS

2.1. Materials.

All components for cell culture were obtained from Invitrogen (Barcelona, Spain). EGTA, BAPTA-AM, Genistein, GF109203X, Pertussis toxin (PTX), SB202190 and KN-62 were obtained from Sigma Aldrich (Alcobendas, Spain). LY294002 and PD98059 were obtained from Calbiochem (Darmstadt, Germany). Progranulin (human recombinant) was obtained from Enzo (Zandhoven, Belgium) and recombinant human/mouse Wnt5a was obtained from R&D Systems (Madrid, Spain). Antibodies used in this study are listed in Table 1. Poly (vinylidene) fluoride (PVDF) membranes were purchased from Bio-Rad (Richmond, CA). The enhanced chemiluminiscence (ECL) system was from Amersham (Uppsala, Sweden.). Other reagents were of molecular biology grade.

2.2. Cell lines

A total of 29 individuals were enrolled in this study: 19 carriers of the c.709-1G>A PGRN gene mutation (7 of them patients of FTLD-TDP and 12 asymptomatic) and 10 control individuals without sign of neurological degeneration. Asymptomatic and control individuals were relatives of patients. All patients were diagnosed as FTD in the Donostia Hospital by applying consensus criteria as published elsewhere (McKhann, et al., 2001). Patients exhibited variable phenotype of initial symptoms. Four of them presented the behavioral variant of frontotemporal dementia (bv-FTD), one progressive non-fluent aphasia and the other corticobasal basal syndrome (CBS). Patients developed a relatively rapidly progressive dementia with features that led to a secondary diagnosis. Notably, this secondary diagnosis was CBS in three of the bv-FTD cases.

The PGRN levels in plasma were strongly reduced in affected and unaffected subjects carrying the c.709-1G>A mutation (a range of 20-61 ng/ml as compared with a range of 90-170 ng/ml in control individuals) (Alquezar, et al., 2012).

All study protocols were approved by the Donostia Hospital and the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. In all cases, peripheral blood samples were taken after written informed consent of the patients or their relatives.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described (Ibarreta, et al., 1998) by infecting peripheral blood lymphocytes with the Epstein Barr virus (EBV). Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml of RPMI-1640 medium that contained 2 mM L-glutamine, 100 µg/ml streptomycin/penicillin and, unless otherwise stated, 10 % (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO₂ incubator at 37°C. Fluid was routinely changed every two days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium. The cellular content of PGRN, determined by Western blotting, was 0.97±0.024 for control individuals, and 0.50±0.021 and 0.48±0.08 for c.709-1G>A PGRN mutation carriers (asymptomatic and FTLD-TDP patients respectively) (see supplementary Fig. 1S).

2.3. PGRN knockdown neuroblastoma SH-SY5Y cell lines

Stable PGRN knockdown neuroblastoma SH-SY5Y cells (Clone # 207) was a generous gift from Drs. Joselin and Wu from the Center for Genetic Medicine (Northwestern University, Chicago, IL US). PGRN knockdown was achieved by using pSUPERIOR RNAi construct as previously described (Gao, et al., 2010). The target sequence of 19 nucleotides targeted against nucleotides 207–226 (#207) of the human PGRN mRNA

was designed. The 64 nt short hairpin RNA sense and antisense primer sequences were 5'-GATCCCCGGCCACTCCTGCATCTTTATTCAAGAGATAAAGATGCAGGAGT GGCCTTTTTGGAAA-3' and

5'-AGCTTTTCCAAAAAGGCCACTCCTGCATCTTTATCTCTTGAATAAAGATG CAGGAGTGGCCGGG-3'.

The sense and antisense primer pairs were annealed and ligated into the pSUPERIOR vector (OligoEngine) according to manufacturer's instructions. The vector control was also stably introduced into SH-SY5Y cells to generate the control cell line.

2.4. Determination of cell proliferation

Cell proliferation was determined by total cell counting, using a TC10™ Automated Cell Counter from Bio-Rad Laboratories, S.A. (Madrid, Spain). EBV immortalized lymphocytes from control and PGRN mutation carriers were seeded at an initial cell concentration of 1×10^6 cells $\times$ ml⁻¹. Cells were enumerated everyday thereafter. In some experiments, cell proliferation was assessed by the 5-bromo-2'-deoxyuridine (BrdU) incorporation method using an enzyme-linked immunoassay kit procured from Roche (Madrid, Spain). Cells (100.000 cells/well for lymphoblast and 15.000 cells/well for neuroblastoma SH-SY5Y cell lines) were seeded in 96-well microtiter plates. Four hours prior to the end of the interval of measurement, BrdU (10 μ M) was added. The cells were fixed with precooled 70% ethanol for 30 min at -20°C and incubated with nucleases following manufacturer's recommendations. Cells were then treated for 30 min at 37°C with peroxidase-conjugated anti-BrdU antibody. Excess antibody was removed by washing the cells three times, followed by the addition of substrate solution. Absorbance was measured at 405 nm with a reference wavelength of 492 nm using a microplate reader.

2.5. Cell cycle analysis

Exponentially growing cultures of cell lines were seeded at an initial concentration of 1×10^6 cells $\times$ ml⁻¹. Cell cycle analysis was performed using a standard method (Krishan, 1975). Cells were fixed in 75% ethanol for 1 h at room temperature. Subsequent centrifugation of the samples was followed by incubation of cells in PBS containing 1 mg/ml of RNase at room temperature for 20 min and staining with propidium iodide (PI; 25 μ g/ml). Cells were analyzed in an EPICS-XL cytofluorimeter (Coulter Científica, Móstoles, Spain). Estimates of cell cycle phase distributions were obtained by computer analysis of DNA content distributions.

2.6. Immunoblotting analysis.

Cells were harvested, washed in PBS and then lysed in ice-cold lysis buffer (50mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1mM sodium orthovanadate, 1mM phenylmethylsulfonylfluride (PMSF), 1mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Mannheim, Germany). 50-100 μ g of protein from cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to PVDF membrane. The membranes were then blocked with 1% Bovine Serum Albumine (BSA) (Sigma) and incubated, overnight at 4°C, with primary antibodies as is indicated in Table 1. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Bio-Rad) and detected with a chemiluminiscent substrate detection system ELC. The specificity of the antibodies was checked by omitting the corresponding primary antibody in the incubation medium. Protein band densities were quantified using Image J software (National Institutes of Health, Bethesda, Maryland, USA) after scanning the images with a GS-800 densitometer from Bio-Rad.

To detect secreted Wnt5a and PGRN proteins, lymphoblasts from control and c.709-1G>A carrier individuals were incubated in RPMI for 24 hours. The conditioned medium was harvested, centrifuged to remove the debris and then concentrated using Amicon Ultra-15 10K centrifugal filter devices (Millipore, Madrid, Spain), treated with proteases inhibitors, PMSF and orthovanadate, and analyzed via Western blotting using antibodies against Wnt5a or PGRN.

2.7. Quantitative real-time PCR

Total RNA was extracted from cell cultures using Trizol reagent (Invitrogen). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2% agarose gel to determine the integrity of 18S and 28S ribosomal RNA. RNA was then treated with DNase I Amplification Grade (Invitrogen). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicates using TaqMan Universal PCR MasterMix No Amperase UNG (Applied Biosystems) reagent according to the manufacturer's protocol. Primers were designed using the Universal ProbeLibrary for Human (Roche Applied Science) and used at a final concentration of 20 μ M. The sequences of the forward and reverse primers used are listed in Table 2. Real time quantitative PCR was performed in the Bio-Rad iQ5 system using a thermal profile of an initial 5-minute melting step at 95 °C followed by 40 cycles at 95 °C for 10 seconds and 60 °C for 60 seconds. Relative messenger RNA (mRNA) levels of the genes of interest were normalized to β -actin expression using the simplified comparative threshold cycle delta CT method ($2^{-[\Delta\text{CTFZD} - \Delta\text{CT Actin}]}$).

2.8. Statistical analyses.

Statistical analyses were performed on GraphPad Prism 5 for Macintosh (La Jolla, CA, USA). All the statistical data are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was estimated by analysis of variance (ANOVA) followed by the Fischer's LSD test for multiple comparisons. Differences were considered significant at a level of $p < 0.05$.

3. RESULTS

3.1. Role of progranulin levels on the proliferative activity of lymphoblasts from control and c.709-1G>A PGRN mutation carrier individuals.

Recent studies from our laboratory indicate that PGRN haploinsufficiency is causally associated to impaired response to serum stimulation (Alquezar, et al., 2011) or withdrawal (Alquezar, et al., 2012) of lymphoblasts bearing the loss-of-function PGRN mutation c.709-1G>A. Here, we present data indicating that treatment of PGRN deficient lymphoblasts with conditioned medium (CM) from control cells or exogenous recombinant human PGRN (rhPGRN) prevented the enhanced proliferation of these cell lines (Fig. 1A,B). It was previously reported that enhanced proliferation of lymphoblasts derived from individuals carrying the c.709-1G>A PGRN mutation was the result of increased levels and activity of CDK6. Thus, we sought to evaluate if exogenous PGRN was able to decrease the enhanced levels of CDK6. As shown in Fig. 1C,D rhPGRN reduced the cellular content of CDK6, as well as the phosphorylation status of the pRb protein, an indirect measurement of CDK6 activity.

3.2. Signaling pathways involved in the serum-mediated stimulation of cell proliferation.

To study the influence of PGRN deficiency on cell proliferation, we investigated the effects of perturbing receptors of different signaling pathways by using Ca^{2+} chelators, inhibitors of protein-kinases and pertussis toxin (PTX). The results of these experiments are summarized in Table 3. Extracellular and intracellular Ca^{2+} levels were reduced by EGTA and BAPTA-AM as described (Huang, et al., 2010). The cell membrane-permeable BAPTA-AM (30 μM) was added to cells incubated in serum-free medium 30 min before replacing the medium by fresh RPMI medium containing 10% FBS. For these experiments, cells were enumerated 24 hours after serum stimulation to avoid

toxicity. Table 3 shows that EGTA significantly inhibited proliferation of control and PGRN deficient cells, however reducing the intracellular concentration of Ca^{2+} by BAPTA, seems to inhibit selectively the serum-mediated enhanced proliferation of lymphoblast from c.709-1G>A PGRN mutation carriers. The average inhibition of cell proliferation, induced by the tyrosine kinase inhibitor, genistein, was similar in control and c.709-1G>A carrying cells (Table 3). Thus, although part of the serum stimulated proliferation is exerted through activation of tyrosin kinases-dependent pathways, these results do not provide an explanation for the enhanced proliferation of c.709-1G>A carriers lymphoblasts. In contrast, treatment of cells with the PKC inhibitor GF109203X or pertussis toxin (PTX) blocked the serum-induced enhanced proliferation of PGRN deficient cells, without affecting significantly the proliferation rate of control cells, thus excluding potential toxic effects of these drugs at the dose used. Moreover, the cell cycle analysis by flow cytometry of control and PGRN deficient cells following treatment with PTX or GF109203X did not reveal the presence of significant sub- G_0/G_1 hypodiploid cells, characteristic of apoptosis/necrosis, in either control or PGRN mutated carriers cells (see supplementary Fig. 2S). It was also observed that the percentage of PGRN deficient cells that reached the S/ G_2M phase is higher than that of control cells (supplementary Fig. 2S). These results suggest that the blockade of the serum-induced increase in the cell number of PGRN deficient cultures by PTX or GF109203X is due to inhibition of cell proliferation rather than the consequence of cytotoxic or apoptotic effects.

Two signaling cascades have emerged as major players in the process of proliferation in many different cells: the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3 kinase (PI3K) pathways (Gerasimovskaya, et al., 2005, Katso, et al., 2001, Meloche and Pouyssegur, 2007). We tested the effects of SB202190, inhibitor of

p38; PD98059, inhibitor of MEK1/2, the upstream activator of ERK1/2, and LY294002 inhibitor of PI3K on the serum-induced proliferation of control and c.709-1G>A carriers. As shown in Fig. 2A (left panel), the inhibition of PI3K/Akt pathway decreased total cell number in cultures of lymphoblasts from control as well as from PGRN deficient individuals, asymptomatic or FLTD patients. In contrast, treatment of cells with the p38 inhibitor, had no consequences in cell number. Interestingly, the specific inhibitor of ERK1/2 selectively blocked the serum-mediated enhancement of total cell number of PGRN deficient lymphoblasts. The possible influence of these drugs on cell viability was analyzed by determining the percentage of hypodiploid sub G₀/1 cells by flow cytometry (Fig. 2A right panel, see also supplementary Fig. 3S). It was observed a moderate increase in the percentage of hypodiploid cells in control and PGRN deficient lymphoblasts in the presence of the PI3K/Akt inhibitor but not in the presence of PD98059 or SB202190. Thus, the lower total cell number in c.709-1G>A carrier cultures observed in the presence of the ERK1/2 inhibitor (Fig. 2A, left panel) must be the result of a truly anti-proliferative action. The effectiveness of these compounds as kinase's inhibitors was confirmed by reduced phosphorylation of the corresponding kinase (Fig. 2 B). Together, these results indicate a specific perturbation of the ERK1/2 pathway in PGRN deficient cells in a PKC-dependent and PTX-sensitive manner.

3.3. Role of ERK1/2 activation in cell proliferation.

Next, we examined the effects of PTX and the inhibitor of PKC on the serum-induced ERK1/2 activation in control and PGRN deficient lymphoblasts. ERK1/2 phosphorylation increased transiently, the maximum effects being 6 hours after serum addition and declined afterwards (results not shown). The addition of serum activated the ERK1/2 pathway in both control and c.709-1G>A PGRN mutation carriers, although the response was enhanced in PGRN deficient cells (Fig. 3A). The increase in

the phosphorylated form of ERKs did not result from increased expression of ERKs, as total ERKs levels were not altered (Fig. 3A). Preincubation of cells with PTX prevented the enhanced ERK1/2 stimulation of c.709-1G>A carrier lymphoblasts without affecting the phosphorylation of ERK in control cells (Fig. 3A, left panel). The PKC inhibitor GF109203X also prevented the serum-mediated ERK1/2 activation (Fig. 3A, right panel) in accordance with the fact that GF109203X and PTX did not have additive effects in inhibiting cell proliferation (Table 3).

We next sought to check whether PTX, and GF109203X are able to block effectively CDK6 levels and enzyme activity. To this end, cells were preincubated with the corresponding drug for 30 minutes before serum stimulation. 24 hours later, cells were harvested to determine by Western blotting the levels of CDK6. Fig. 3B shows how PTX (left panel) or GF109203X (right panel) treatments were effective in decreasing the content of CDK6 of lymphoblasts carrying the c.709-1G>A PGRN mutation up to levels similar to those of control cells.

3.4. Wnt signaling in PGRN deficient lymphoblasts.

Bearing in mind that a PTX-sensitive G-protein coupled receptors (GPCR) signaling pathway seems to be exacerbated in PGRN deficient cells, we focused on the Wnt pathway based on a recent report that showed increased expression of Frizzled (FZD) receptors in a cell model of PGRN deficiency (Rosen, et al., 2011). These authors performed weighted gene expression network analysis finding robust changes in expression of genes of the cell cycle and ubiquitination in PGRN deficient neural stem cells in accordance with the human pathology, revealing an unexpected role of Wnt signaling pathway. The ligands for frizzled receptors are the Wnt family of glycosylated lipid-modified cysteine rich proteins (Janda, et al., 2012). So far, at least 19 members of Wnt have been identified in mammals (Miller, 2002). It is known that

some members signal through the so-called canonical pathway involving β -catenin while others, mainly the Wnt5a class, are independent of β -catenin (Huelsken and Birchmeier, 2001). Non-canonical signaling correlates with increased cytosolic Ca^{2+} levels and elevated CaMKII and PKC activities (Kikuchi, et al., 2012, Kuhl, et al., 2000). It also activates small and heterotrimeric G proteins (Niu, et al., 2012). Interestingly, a recent report demonstrated that signaling through Wnt5a is PTX-sensitive (Kilander, et al., 2011), indicating the involvement of a $\text{G}_{\alpha i}$ GPCR. On these grounds, we first determined the expression levels of some FZD receptors in control and PGRN mutation carrier lymphoblasts. By using real time quantitative PCR, we found that lymphoblastoid cells express FZD2, FZD3, FZD4, and FZD5 receptors, being the FZD4 and FZD5 mRNA the more abundant transcripts. We did not find differences in the expression levels of any of these receptors between control and PGRN deficient cells (Table 4). In contrast, we found increased cellular Wnt5a protein levels in lymphoblasts from asymptomatic and FTLD-TDP patients when compared with those of control cells (Fig. 4A). The basal and Wnt5a-induced activation of cell proliferation, assessed by the rate of BrdU incorporation into DNA, is presented in Fig. 4B. It is shown that exogenous Wnt5a selectively increased the proliferative activity of control lymphoblasts up to the level found in PGRN deficient cells, which express higher levels of Wnt5a (Fig. 4B). As shown in Fig. 4C, the lymphoblasts bearing the c.709-1G>A PGRN mutation display enhanced basal CaMKII activation, as monitored by increased basal levels of phospho-CaMKII. The treatment of cells with exogenous Wnt5a increased the CaMKII activity in control cells without affecting the phosphorylation status of CaMKII of PGRN deficient lymphoblasts (Fig. 4C). These results suggest that increased Wnt5a/ Ca^{2+} signaling might be involved in regulating enhanced proliferation in peripheral cells from FTLD patients. Supporting this

possibility is the fact that increased proliferation of PGRN deficient lymphoblasts was prevented by chelating intracellular Ca^{2+} (Table 3). Moreover, using KN-62, a specific inhibitor of the Wnt5a/ Ca^{2+} pathway downstream mediator CaMKII, also significantly reduced cell proliferation (Fig. 4D, left panel) and ERK1/2 activity (Fig. 4D, right panel) of PGRN deficient lymphoblasts.

We next evaluated the dependence of Wnt5a-induced stimulation of proliferation on the activation of the $G_{\alpha i}$ /ERK1/2/CDK6 signaling pathway. To this end, we determined whether the endogenous and Wnt5a-induced enhanced cell proliferation was sensitive to PTX treatment. As shown in Fig 5A, the effect of exogenous Wnt5a on proliferation of control lymphoblasts was abrogated in the presence of PTX. Moreover, PTX was also effective in preventing the increased proliferation of lymphoblasts carrying the PGRN c.709-1G>A mutation (Fig 5A). In Fig. 5B it is shown that exogenous Wnt5a increased ERK1/2 activity, elevated CDK6 levels and the phosphorylation of the pRb protein in control cells, in a PTX-sensitive manner.

Two different experimental approaches were used to confirm that increased Wnt5a signaling is responsible for the enhanced proliferation of lymphoblasts bearing the c.709-1G>A PGRN mutation. First, we measured the levels of secreted Wnt5a and PGRN, observing significant reciprocal changes in the levels of these proteins in conditioned medium (CM) from PGRN deficient cells, and studied the effect of this CM on proliferation of control lymphoblasts. Second, we treated control and c.709-1G>A PGRN bearing lymphoblasts with a neutralizing antibody against Wnt5a or with an irrelevant IgG. Fig. 6A shows that the CM from PGRN deficient cells mimicked the effect of exogenous Wnt5a increasing proliferation of control cells reaching values similar to those found in lymphoblasts bearing the PGRN mutation. On the contrary, the addition of anti-Wnt5a antibody to PGRN-deficient cells abrogated the serum-enhanced

cell proliferation of these cell lines (Fig.6B). The anti-Wnt5a antibody was also able to blunt the enhanced Wnt5a signaling in PGRN deficient lymphoblasts, as it decreased the levels of pCaMKII, pERK1/2 and CDK6 (Fig. 6C). Taken together, these results indicate that PGRN haploinsufficiency is associated with enhanced levels and signaling of Wnt5a.

3.5. PGRN deficiency and Wnt5a signaling in human neuroblastoma SH-SY5Y cells.

We addressed the issue as to whether PGRN deficiency would impact Wnt5a signaling in neuronal cells. We used PGRN knockdown human neuroblastoma SH-SY5Y cells to determine the cellular levels of Wnt5a protein and to analyze the effect of reduced PGRN on Wnt5a signaling. As expected, these cells displayed lower levels of PGRN (Fig. 7A). In parallel, we observed increased cellular content of Wnt5a protein (Fig. 7B). PGRN deficiency seems to activate the Wnt5a signaling pathway in neuroblastoma SH-SY5Y cells, as indicated by higher basal levels of p-CaMKII and ERK1/2 compared with those of control cells (Fig. 7C,D). Treatment of control cells with exogenous Wnt5a protein induced the activation of these two enzymes to levels similar to those of PGRN KD SH-SY5Y cells (Fig. 7C,D). Moreover, both increasing endogenous or exogenous Wnt5a levels resulted in enhanced incorporation of BrdU into DNA in SH-SY5Y neuroblastoma cells (Fig. 7E).

4. DISCUSSION

Mounting evidence suggested that aberrant cell cycle activation in postmitotic neurons contributes to neuronal pathology and neurodegeneration (Bonda, et al., 2010, Wishart, et al., 2006). It was proposed that aberrant reactivation of cell cycle might render a higher vulnerability of neurons to other detrimental insults according to the so-called “two-hit hypothesis” (Zhu, et al., 2004). We previously addressed the issue as to whether PGRN deficiency impairs the control of cell cycle by performing comparative analysis of the proliferative activity and cell survival/death mechanisms in immortalized lymphocytes from control and individuals with a null PGRN mutation (c.709-1G>A) either asymptomatic or diagnosed of FTLD-TDP (Alquezar, et al., 2012, Alquezar, et al., 2011). We reported neoplastic-like features of lymphoblasts from FTLD-TDP patients associated with increased levels and activity of CDK6 (Alquezar, et al., 2011). Here we have studied the underlying signaling pathways involved in the enhanced serum-mediated proliferative response of PGRN deficient cells. Our results suggest that non-canonical Wnt5a signaling could be overactivated by PGRN haploinsufficiency. This finding is in line with a recent report in which a genome-wide analysis of Wnt regulated transcriptional network indicated that Wnt and PGRN reciprocally regulate each other, therefore providing a connection between Wnt signaling and FTLD-TDP pathology (Wexler, et al., 2011). The data herein presented suggest that Wnt5a signaling could be aberrantly activated in lymphoblasts bearing the PGRN mutation c.709-1G>A. Several lines of evidence support this possibility, i) addition of exogenous Wnt5a to control lymphoblasts enhanced the proliferative activity up to the levels found in PGRN deficient cells; ii) exogenous Wnt5a was effective in increasing CDK6 levels in control cells; iii) conditioned medium from PGRN deficient cells showed enhanced levels of Wnt5a; iv) addition of conditioned medium from PGRN deficient cells increased

proliferation of control cells. v) blocking Wnt activation in PGRN deficient cells with an antibody against Wnt5a normalized the proliferative activity of PGRN deficient cells.

The role of Wnt5a on cell proliferation is complex. Wnt5a has been found to promote the proliferation of human glioblastoma (Yu, et al., 2007), lung fibroblasts (Vuga, et al., 2009), and B cell lymphopoiesis in bone marrow cell culture (Malhotra, et al., 2008), but to suppress the proliferation of B cells in Wnt5a KO mice (Liang, et al., 2003). Based on these studies, the effects of Wnt5a on cell proliferation seem to depend on cell type. We report here a pro-proliferative action of exogenous Wnt5a in control lymphoblasts, as well as enhanced proliferative activity in PGRN mutant cells when compared with control cells. Increased cellular and secreted levels of Wnt5a were found in lymphoblasts derived from individuals harboring the c.709-1G>A PGRN mutation regardless of the clinical status thus suggesting that the increased Wnt5a signaling is probably an early etiologically relevant event during FTLD-TDP development.

Our data indicate that Wnt5a signals through a G_{ai} -PLC-PKC-ERK1/2 cascade. The increased cell proliferation observed in PGRN deficient cells is abrogated in the presence of pertussis toxin. Moreover the effects of exogenous Wnt5a in control cells are also sensitive to PTX inhibition. This observation is in accordance with previous reports that indicated that Wnt5a stimulation involves activation of heterotrimeric G_{ai} proteins in mammalian cells (Kilander, et al., 2011, Wilson, et al., 2010). PGRN deficiency is accompanied by enhanced stimulation of the ERK1/2 pathway, most likely through the non-canonical activation of Wnt5a as indicated by the observed increased levels of p-CaMKII and evidences of PKC activation in lymphoblasts from c.709-1G>A PGRN mutation carriers. Moreover, inhibition of these two kinases by the specific inhibitors KN-62 and GF109203X respectively resulted in the abrogation of the

enhanced proliferative response of PGRN-deficient cells. The proposed scenario is represented schematically in Fig 8. $G_{\alpha i}$ protein-dependent Wnt5a signaling to ERK1/2-mediated increased proliferation had been previously described in microglia cells (Halleskog, et al., 2012). The molecular mechanism by which ERK1/2 regulates the expression levels and kinase activity of CDK6 in PGRN-deficient lymphoblasts cannot be ascertained by the present results. The expression of CDK6 is negatively regulated by TDP-43 (Ayala, et al., 2008). Several reports indicate that PGRN deficiency induced mislocalization of TDP-43 protein leading to a loss of the DNA-RNA binding function of the protein (Dormann, et al., 2009, Zhang, et al., 2007). Indeed an increase in the cytosolic content of TDP-43 protein could be observed in c.709-1G>A bearing lymphoblasts associated with increased levels of CDK6 (Alquezar, et al., 2011). Recently, the ERK1/2 pathway has been found to play an important role in TDP-43 nuclear clearance and cytoplasmatic accumulation of the protein in a cell model of induced stress in neurons and in Hela cells (Parker, et al., 2012). Thus it is tempting to speculate that ERK1/2-mediated increased CDK6 expression levels and cell proliferation in PGRN deficient cells could be the result of enhanced exit of TDP-43 from the nucleus.

In summary, we provided evidence that PGRN haploinsufficiency aberrantly increased non-canonical, $G_{\alpha i}$ dependent-Wnt signaling leading to activation of ERK1/2-mediated stimulation of proliferation of lymphoblasts derived from individuals bearing a FTLD-associated PGRN mutation.

Although FTLD-TDP associated changes detected in peripheral cells might not fully reflect those in FTLD-TDP brain, it is evident that besides neuronal damage there are also peripheral aspects of the disease. A close relationship seems to exist between the state of the immune system, and particularly lymphocytes, and some psychiatry

disorders including Alzheimer's disease (AD) (Gladkevich, et al., 2004). Recent work indicated a possible role of activation of the non-canonical Wnt pathway in AD pathogenesis by orchestrating the neuroinflammatory response (Li, et al., 2011). Our results show that Wnt5a signaling is also enhanced in human neuroblastoma SH-SY5Y after silencing of the PGRN gene. Thus, it is possible that a mechanism involving neuroinflammatory cytokines that link the peripheral immune and nervous systems could influence neuronal survival in FTLTDP. Aberrant activation of Wnt signaling is associated with several neurodegenerative disorders including AD, ALS and Parkinson's disease (Chen, et al., 2012, da Cruz e Silva, et al., 2010, Inestrosa and Arenas, 2010, L'Episcopo, et al., 2012). Assuming that the PGRN deficiency and Wnt5a-mediated cell cycle disturbances reported here could be peripheral signs of the disease, our results suggest that modulators of Wnt signaling may play a critical role in the pathogenesis of FTLTDP, and therefore Wnt5a signaling could be an important target for developing novel therapies.

Acknowledgements

This work has been supported by grants from Ministerio de Economía y Competitividad (SAF2011-28603) and Fundación Ramón Areces to AM-R. Dr. López de Munain received research support from the Basque Government, Spain (SAIO11.PR11BN002). We thank Drs. Joselin and Wu for providing the PGRN KD SH-SY5Y cells. We would like to thank to all patients, their families and clinicians involved in this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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TABLES:

Table 1.
SUMMARY OF ANTIBODIES USED FOR WESTERN BLOTTING.

Antibody name	Company	Dilution
β -actin (ACTBD11B7)	Santa Cruz (sc-81178)	1:500
Phospho-Akt(Ser473)	Cell Signaling (#9271)	1:1000
Akt1 (C-20)	Santa Cruz (sc-1618)	1:1000
Phospho-CaMKII (Thr286)	Cell Signaling (#3361)	1:500
CaMKII	Cell Signaling (#3362)	1:500
Cdk6 (C-21)	Santa Cruz (sc-177)	1:1000
Anti-Granulin (EPR3781)	Abcam (ab108608)	1:500
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) XP	Cell Signaling (#4370)	1:2000
p44/42 MAP Kinase	Cell Signaling (#9102)	1:2000
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP	Cell Signaling (#4511)	1:1000
p38 MAPK	Cell Signaling (#9212)	1:1000
Rb (C-15)	Santa Cruz (sc-50)	1:500
α -tubulin (B-5-1-2)	Santa Cruz (sc-23948)	1:1000
Anti-Wnt5a	Abcam (ab72583)	1:500

TABLE 2
SEQUENCES OF OLIGODEOXYRIBONUCLEOTIDE PRIMERS USED FOR QUANTITATIVE
REAL-TIME PCR

Gene	Primer Sequence	
	Forward (5'→3')	Reverse (5'→3')
FZD2	GGTGTCTGGTGGCCTACAT	GAGAAGCGCTCGTTGCAC
FZD3	ACAGCAAAGTGAGCAGCTACC	CTGTAACTGCAGGGCGTGTA
FZD4	TTCACACCGCTCATCCAGTA	TGCACATTGGCACATAAACA
FZD5	TCTTCACGCTGCTCTACACG	GGTAGTGCTGCTCGTACAGGT
β-ACTIN	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG

Probes were designed using the Universal ProbeLibrary form Human (Roche Applied Science)

TABLE 3

INFLUENCE OF CALCIUM CHELATORS, MAPK AND PI3K INHIBITORS ON
PROLIFERATION OF CONTROL AND c.709-1G>A CARRIERS LYMPHOBLASTS

Cell Treatment	% inhibition of cell growth		
	Control	Asymptomatic	FTLD Patients
EGTA (1mM)	24±4	35±7	34±5
BAPTA (30 µM)	0±6	31±4*	34±5*
Genistein (1 µM)	37±4	33±3	28±4
GF109203X (50nM)	4±4	32±8*	29±6*
PTX (0.2ng/ml)	6±6	29±4*	29±4*
PTX+ GF109203X	8±4	30±6*	35±2*

Cells were seeded at an initial density of 1×10^6 /ml on day 0 and treated with the indicated concentrations of EGTA, BAPTA-AM, genistein, GF109203X, and pertussis toxin (PTX). BAPTA-AM was added to cells incubated in serum-free medium 30 min before replacing the medium by fresh RPMI medium containing 10% FBS. To avoid toxicity in the incubations with EGTA or BAPTA-AM, cell proliferation was determined 24 hours after serum stimulation; otherwise proliferation was determined 72 hours after drug addition. Values shown are the mean $\pm$ SEM for seven observations. * $p < 0.05$, significantly different from control cells.

TABLE 4.
RELATIVE FZD mRNA ABUNDANCE

Gene	c.709-1G>A		
	Control	Asymptomatic	FTLD Patients
FZD2	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
FZD3	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.5
FZD4	1.0 ± 0.1	0.9 ± 0.2	1.1 ± 0.4
FZD5	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.2

Immortalized lymphoblasts from control and c.709-1G>A individuals were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ and cultured for 24 hours in RPMI medium containing 10% FBS. Cells were collected and subjected to RT-qPCR. Relative mRNA levels of the FZD(2,3,4,5) genes were normalized to β -actin expression, and values for control cells were set as one. Values shown are the mean $\pm$ SEM for four different cell lines.

Figure Legends:

Fig. 1

Influence of PGRN levels on the proliferative activity of human lymphoblasts

A: Immortalized lymphocytes from control and c.709-1G>A PGRN mutation carriers, asymptomatic or FTLT-TDP patients, were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ and were incubated for 72 h in the absence (white bars) or in the presence (black bars) of conditioned medium (CM) from control cells (left panel) or with 100ng/ml of rhPGRN (right panel). Cell proliferation was determined by counting the cells excluding trypan blue using a TC10TM Automated Cell Counter. Values shown are the mean $\pm$ SEM for six observations carried out with different cell lines from control and PGRN mutated carriers. * $p < 0.01$ significantly different from control cells; + $p < 0.01$ significantly different from untreated cells. B: Representative immunoblots showing the effects of rhPGRN on CDK6 levels (left panel) and pRb phosphorylation status (right panel). The densitometric data represent the mean $\pm$ SEM of six determinations.

Fig. 2

Effects of PI3K/Akt and MAP kinases inhibitors on the serum-enhanced proliferation of PGRN deficient lymphoblasts.

A: Immortalized lymphocytes from control and c.709-1G>A PGRN mutation carriers, asymptomatic or FTLT-TDP patients, were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ and were incubated for 72 h in RPMI medium containing 10% FBS, in the absence or in presence of 20 μM Ly294022, 20 μM PD98059 or 10 μM SB20219. Aliquots were taken for cell counting (left panel) and for determination of the percentage of hypodiploid sub $G_0/1$ cells by flow cytometry (right panel). Data shown are the mean $\pm$ SEM of seven determinations carried out with different cell lines * $p < 0.01$ significantly different from

control cells, +p<0.01 significantly different from untreated cells. B: Representative immunoblots from control and c.709-1G>A carriers individuals showing the effect of each of these drugs on its specific target six hours after their administration. The densitometric data represent the mean $\pm$ SEM of seven determinations, *p<0.05 significantly different from control cells, +p<0.05 significantly different from untreated cells.

Fig. 3

Activation of ERK1/2 pathway in control and PGRN deficient lymphoblasts.

Effects of PTX and GF109203X.

A: Effects of PTX (left panel) and GF109203X (right panel) on ERK1/2 activation after serum addition. Immortalized lymphocytes from control and c.709-1G>A PGRN mutation carriers, asymptomatic or FTLTD-TDP patients, were seeded at an initial density of $1 \times 10^6 \text{ ml}^{-1}$, and were incubated in RPMI medium containing 10% FBS in the absence (white bars) or in the presence of 0.2 ng/ml PTX or 50nM GF109203X (black bars) and harvested 6 h later for cell extracts preparation. B: effects of PTX or GF109203X on CDK6 levels in control and PGRN deficient lymphoblasts. Representative immunoblots are shown. Densitometric measurements were performed on individual immunoblots and values indicate protein levels normalized to the corresponding β -actin levels $\pm$ SEM for six different experiments. *p<0.05 significantly different from control cells, +p<0.05 significantly different from untreated cells

Fig. 4

Wnt5a signaling is enhanced in c.709-1G>A PGRN mutation carriers.

A: Immortalized lymphocytes from control and c.709-1G>A PGRN mutation carriers, asymptomatic or FTLT-TDP patients, were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ and incubated in RPMI medium 24 h before cells were collected to prepare cell extracts for Western blotting. A representative blot is shown while below the densitometric analyses represent the mean $\pm$ SEM of different observations carried out in six cell lines from each type. B: Proliferative response of control and PGRN deficient cells in the presence or in the absence of 0.1 $\mu\text{g/ml}$ recombinant human Wnt5a. 100.000 cells/well were seeded in 96-well plates, 24 h after, cells were pulsed with 10 μM BrdU for 4 h. DNA synthesis was assessed by BrdU incorporation method according to the manufacturer's instructions. Proliferation was expressed as absorbance of stimulated minus that of nonstimulated cultures. Each bar represents the mean $\pm$ SE of six independent experiments performed in triplicate. C: Effect of Wnt5a addition on CaMKII activity. Representative immunoblots showing phosphorylated and total CaMKII are presented. Densitometric analyses represent the mean $\pm$ SEM of six determinations carried with different cell lines. D: Effect of the treatment with KN-62 on proliferation (left panel) and in the activation of ERK1/2 (right panel) in control and PGRN deficient lymphoblasts. Cells were incubated during 72 h in the absence or in presence of 1 μM KN-62 for cell counting. Cell extracts to determine ERK1/2 activation were prepared 6 h after drug administration. Data shown are the mean $\pm$ SEM for six determinations carried out with different cell lines. * $p < 0.05$ significantly different from control cells, + $p < 0.05$ significantly different from untreated cells.

Fig. 5

Effects of exogenous Wnt5a on cell proliferation, ERK1/2 activity, CDK6 levels and pRb phosphorylation status.

A: Immortalized lymphocytes from control and c.709-1G>A PGRN mutation carriers, asymptomatic or FTLT-TDP patients, were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ and were incubated for 72 h in RPMI medium containing 10% FBS, in the absence or in the presence of 0.1 $\mu\text{g/ml}$ Wnt5a, alone or in combination with 0.2 ng/ml of PTX. Values shown are the mean $\pm$ SEM for six determinations carried out with different cell lines. B: For cell extracts, cells were harvested 6 h after drug addition. Representative immunoblots show the effects of Wnt5a decreasing the ERK1/2 phosphorylation, CDK6 levels and pRb phosphorylation in control cells in a PTX-dependent manner. Below it is shown the densitometric measurements of individual immunoblots for ERK1/2 and CDK6 proteins, normalized to the corresponding total ERK1/2 or β -actin respectively. Values shown are the mean $\pm$ SEM for six individual experiments. * $p < 0.05$ significantly different from control cells, + $p < 0.05$ significantly different from untreated cells. pp= the hyperphosphorylated form of pRb.

Fig. 6

Influence of Wnt5a signaling activity in the proliferative response of lymphoblasts from control or c.709-1G>A PGRN mutation carriers.

A: Immortalized lymphocytes from control and c.709-1G>A PGRN mutation carriers were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$. Control cells were incubated for 72 h in conditioned medium (CM) from PGRN deficient lymphoblasts. Representative immunoblots for secreted levels of Wnt5a and PGRN in the CM from PGRN deficient cells are shown. Below it is shown the proliferative activity of control cells incubated in

the presence of CM. For comparison, proliferation of lymphoblasts from asymptomatic or FTLT-TDP patients is shown. Cell proliferation was determined by cell counting. Values shown are the mean $\pm$ SEM for six observations carried out with different cell lines from control and PGRN mutated carriers. B: Effects of a neutralizing antibody against Wnt5a in cell proliferation. Lymphoblasts from control and c.709-1G>A PGRN mutation carriers individuals were incubated in the absence or in the presence of 0.2 μ g/ml of anti-Wnt5a antibody. Aliquots were taken 72 h later for cell counting. Values shown are the mean $\pm$ SEM of four determinations carried out with different cell lines. C: Effects of anti-Wnt5a antibody on signaling through the Ca²⁺/CaMKII/ERK1/2/CDK6 pathway. Representative immunoblots showing the effects of anti-Wnt5a antibody in the phosphorylation status of CaMKII and ERK1/2, and CDK6 levels are shown in the left panel while densitometric analyses are presented in the right panel. Values shown are the mean $\pm$ SEM for six individual experiments. *p<0.05 significantly different from control cell +p<0.05 significantly different from untreated cells.

Fig. 7

PGRN deficiency and Wnt5a signaling in human neuroblastoma SH-SY5Y cells. A, B: Whole cell extracts of SH-SY5Y clones expressing either the control vector, or a target sequence of human PGRN mRNA, were analyzed for the expression of PGRN and Wnt5a by Western blotting using specific antibodies. β -actin levels were analyzed as loading controls. Values shown for the densitometric analyses are the mean $\pm$ SEM for four individual experiments. *p<0.05 significantly different from control cells. C, D: Effect of the treatment with exogenous Wnt5a in the activation of pCaMKII and ERK1/2 respectively in control and PGRN knockdown cells. Cell extracts were

prepared 6 h after drug administration (0.1 µg/ml). Data shown are the mean ± SEM for four individual experiments. *p<0.05 significantly different from control cells. E: Proliferative response of control and PGRN knockdown cells in the presence or in the absence of recombinant human Wnt5a. 15.000 cells/well were seeded in 96-well plates, 24 h after cells were pulsed with 10 µM BrdU for 4 h. DNA synthesis was assessed by BrdU incorporation method according to the manufacturer's instructions. Proliferation was expressed as absorbance of stimulated minus that of nonstimulated cultures. Data shown are the mean ± SEM for four determinations carried out with different cell lines. *p<0.05 significantly different from control cells, +p<0.05 significantly different from untreated cells.

Fig. 8

Diagram summarizing the influence of PGRN haploinsufficiency in increasing non-canonical Wnt5a signaling, Ca²⁺, and PKC-dependent PTX-sensitive leading to enhanced cell proliferation.

PGRN deficient cells appear to secrete increased levels of Wnt5a. The binding of Wnt5a to a Gα_{i/o} protein coupled receptor activates the release of Ca²⁺ from intracellular stores, which in turn stimulates CaMKII and PKC, upstream modulators of ERK1/2 pathway and cell proliferation. Treatment of lymphoblasts bearing the c.709-1G>A PGRN mutation with the ERK1/2 inhibitor PD98059 prevented the serum-mediated enhancement of cell proliferation.

AUTHOR'S RESPONSE TO THE REVIEWERS

Ms. NBA-13-58

“PGRN haploinsufficiency increased Wnt5a signaling in peripheral cells from FTL D-progranulin mutation carriers”.

C. Alquézar, N. Esteras, A. de la Encarnación, A. Alzualde, F. Moreno, A. Lopez de Munain, A. Martín-Requero

Response to Reviewers

We thank the Reviewers for their excellent and constructive criticism that have helped to improve our study. We have deal with the reviewers' concerns as outlined below.

Reviewer #1

1. Following the reviewer's recommendation, we have now included quantitative data and statistics in former Figs 1 and 3, now Figs 1 and 2.
2. We understand the reviewer's concern about considering cell counts as cell proliferation based in previous work from our lab. For the revised version of our Ms, we have performed additional experiments. First, to rule out toxic effects of the kinases's inhibitors used in former Fig. 3, we have analyzed the cell cycle status. The new data is provided in the new Fig. 2A (right panel), and supplementary Fig. 3S. As shown in Fig. 2A, (right panel), no signs of increased fraction of hypodiploid cells, indicative of necrosis/apoptosis, were detected in control and c.709-1G>A carriers lymphoblasts, in the absence or in the presence of the concentration indicated of PD98059, or SB202190. Thus, the effect of the ERK1/2 inhibitor, PD98059, decreasing the total cell number of PGRN deficient lymphoblasts cultures can be considered as truly anti-proliferative. Second, we have analyzed the rate of BrdU into DNA, as an index of cell proliferation, of control and PGRN mutation carriers in the absence or in the presence of exogenous Wnt5a. The new data are presented in Fig. 4B of the revised version of our Ms.
3. Following the reviewer's suggestion, we have preformed a set of experiments to assess the capacity of neutralizing antibodies anti-Wnt5a to block the cellular response of control, asymptomatic and FTL D-TDP patients. The results obtained are presented in the Fig. 6 of the revised version of the Ms.

Minor points:

1. The legend of former Fig. 1 has been corrected.
2. As suggested former Fig. 2 is now included as supplementary Fig 2S.

3. We have provided the source of all antibodies used (Table 1 of the revised version of our Ms).
4. We thank the reviewer for indicating the missing Y-axis label in former Fig. 6. This has been corrected in the revised version of our Ms.

Reviewer #2

1. We understand the reviewer's concern about cell-counting as a measure of cell proliferation. However, experiments presented in former Fig. 2 were conducted to precisely rule out the possibility that increased total cell number in PGRN-deficient lymphoblast were due to lower rates of apoptosis/necrosis. Since we didn't observed an increase in the number of hypodiploid cells in asymptomatic or FTLT-TDP lymphoblasts in the presence of PTX or GF109203X, we concluded that they act as truly antiproliferative drugs. Nonetheless, we have performed additional experiments to rule out possible toxic effects of drugs used in former Fig. 3. The results obtained are presented in a new supplementary figure (Fig.3S). In addition, we have measured the rate of BrdU incorporation into DNA in control and PGRN deficient lymphoblasts. As shown in the new Fig.4B. c.709-1G>A lymphoblasts show increased rates of BrdU incorporation when compared with control cells. Moreover, the addition of exogenous Wnt5a to control cells induced an increase on BrdU incorporation, thus indicating increased cell proliferation.
2. Cellular content of PGRN was determined by Western blotting in lymphoblasts from control, asymptomatic and FTLT-TDP subjects. We have included the densitometric analyses in the Material and Methods section in the revised version of our Ms (page 7), and a supplementary figure (Fig.1S) is now provided
3. We appreciate this suggestion. We indeed consider important to replicate our findings in a neuronal cell. Since most of the PGRN mutations, including the c.709-1G>A are null mutations, we used a stable clone of SH-SY5Y, which expressed reduced levels of PGRN. These cells were a generous gift from Drs. Joselin and Wu from the Northwestern University Feinberg School of Medicine, Chicago. As expected the knockdown PGRN SH-SY5Y cells show reduced levels of PGRN. We found that these KO cells have higher content of Wnt5a when compared with control cells (expressing the empty vector). Once more, this observation validates some how the use of peripheral tissues from patients as surrogate to study pathophysiological features of diseases of the CNS. We have performed some experiments to assess the activation of CaMKII/ERK1/2 signaling pathway in the absence and presence of exogenous Wnt5a. In addition, we have determined the effect of Wnt5a in the rate of incorporation of BrdU into DNA of control and KO SH-SY5Y cells. The results obtained have been summarized in the Fig. 7 of the revised version of our Ms.
4. We have tried to be consistent in the use of FTLT-TDP

Authors Statement

On behalf of my colleagues I do hereby certify that none of the co-authors have potential conflicts of interest. In addition, I certify that all the authors have reviewed the manuscript and approved its content.

All study protocols were approved by the Spanish Council of Higher Research, Institutional Review Board and are in accordance with National and European Union guidelines.

The data contained in the manuscript have not been previously published. It has not been submitted to other journal, nor it will be submitted elsewhere.

Fig. 1[Click here to download high resolution image](#) **Fig.1**

Fig. 2[Click here to download high resolution image](#) **Fig. 2**

Fig. 3[Click here to download high resolution image](#)**A** **B** **Fig. 3**

Fig. 5[Click here to download high resolution image](#) **Fig. 5**

Fig. 6[Click here to download high resolution image](#) **Fig. 6**

Fig. 7

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