Progranulin Reduction Is Associated With Increased Tau Phosphorylation in P301L Tau Transgenic Mice

Masato Hosokawa, PhD, Tetsuaki Arai, MD, PhD, Masami Masuda-Suzukake, PhD, Hiromi Kondo, Takashi Matsuwaki, DVM, PhD, Masugi Nishihara, DVM, PhD, Masato Hasegawa, PhD, and Haruhiko Akiyama, MD, PhD

Abstract
Granulin (GRN) mutations have been identified in familial frontotemporal lobar degeneration patients with ubiquitin pathology. GRN transcript haploinsufficiency is proposed as a disease mechanism that leads to the loss of functional progranulin (PGRN) protein. Thus, these mutations are strongly involved in frontotemporal lobar degeneration pathogenesis. Moreover, recent findings indicate that GRN mutations are associated with other neurodegenerative disorders with tau pathology, including Alzheimer disease and corticobasal degeneration. To investigate the potential influence of a decline in PGRN protein on tau accumulation, P301L tau transgenic mice were interbred with GRN-deficient mice, producing P301L tau transgenic mice harboring the GRN hemizygote. Brains were collected from 13- and 19-month-old mice, and sequential extraction of proteins, immunoblotting, and immunohistochemical analyses were performed. Immunoblotting analysis revealed that tau phosphorylation was accelerated in the Tris-soluble fraction of 19-month-old mice compared with GRN-hemizygote mice. Brains were collected from 13- and 19-month-old mice, and sequential extraction of proteins, immunoblotting, and immunohistochemical analyses were performed. Immunoblotting analysis revealed that tau phosphorylation was accelerated in the Tris-soluble fraction of 13-month-old mice and in the sarcosyl-insoluble fraction of 19-month-old P301L tau/GRN hemizygotes compared with those in fractions from P301L tau transgenic mice. Activity of cyclin-dependent kinases was also upregulated in the brains of P301L tau/GRN hemizygote mice. Although the mechanisms involved in these findings remain unknown, our data suggest that a reduction in PGRN protein might contribute to phosphorylation and intraneuronal accumulation of tau.

Key Words: Alzheimer disease, Granulin, Phosphorylation, Progranulin, Tau.

INTRODUCTION
Progranulin (PGRN) is a growth factor that is encoded by a single gene on chromosome 17q21. It is a 593-amino acid cysteine-rich protein with a signal peptide (17 amino acids) and highly conserved 7.5 tandem granulin repeats of a 12-cysteinyln motif. It is involved in the regulation of multiple functions, including neuronal cell growth (1, 2), wound healing (3, 4), and inflammation (5). It has also been strongly linked to tumorigenesis (6). Moreover, PGRN has a chemotactic effect for microglia (7). In 2006, granulin (GRN) null mutations were identified in familial frontotemporal dementia linked to chromosome 17q21 with tau-negative ubiquitin-positive inclusions. GRN is located at 1.7 Mb centromeric of the MAPT (tau) gene (8, 9). Many mutations, including frame shift by insertion and deletion or substitution of a nucleotide that generate premature termination codons, have been reported. GRN transcript haploinsufficiency has been proposed as the disease mechanism that leads to the loss of functional PGRN protein. Premature stop codons are not translated into the mutant transcript because translation is blocked by nonsense-mediated RNA decay. The mutation in the signal peptide may cause mislocalization of PGRN in a protein secretion pathway or PGRN loss of function by impairment of PGRN transport (10, 11). Thus, these mutations are strongly involved in frontotemporal dementia pathogenesis.

Loss-of-function GRN mutations have been confirmed in patients clinically diagnosed as having Alzheimer disease (AD) (12–18). Of these, R535X (c.1603 C > T) and Null (IVS0 +5G > C) mutations were originally identified in frontotemporal lobar degeneration (FTLD)-GRN but were also found in AD (12). Two further mutations found in AD, p.Cys139Arg (c.415 T > C) and p.Pro451Leu (c.1352C > T), were indicated to be pathogenic based on evolutionary conservation and silico protein modeling (19). Mutations, p.Gly35Arg (c.103G > A) (20), and a single base pair deletion (c.154delA) were also found in AD, and the latter was shown to cause a frame shift (p.Thr52HisfsX2), creating a premature stop codon. Similarly, a single base pair deletion (c.154delA) was also found in AD, and the latter was shown to cause a frame shift (p.Thr52HisfsX2), creating a premature stop codon.

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stop codon (21). The rs5848 (3’UTR +78C > T) variant in the 3’ untranslated region (UTR) of GRN is known to reduce GRN mRNA levels in the brain and peripheral mononuclear cells in patients. The rs5848 variant was also found in AD (22) and is associated with a risk for AD (23). Granulin mutations have also been found in another tauopathy, corticobasal syndrome (24); they include a novel splice donor site mutation in the GRN gene p.Val200GlyfsX18 (IVS7+1G > A) (25), p.Val279GlyfsX5 (IVS8-G > C) (10), p.Thr272SerfsX10 (c.813_816delCACT) (26), and p.Ala9Asp (c.26C > A) (27). Furthermore, tau pathology, in addition to TDP-43 pathology, was reported to be found in most patients in 2 families harboring a GRN mutation (28).

These findings suggest that the decline or dysfunction of PGRN may contribute to tau abnormalities that lead to tau pathology by an unknown mechanism. To elucidate these issues, we produced P301L tau transgenic mice harboring the GRN hemizygote (Tau/GRN(+/-)) by interbreeding P301L tau transgenic mice with GRN-deficient mice and analyzed whether the GRN reduction affects phosphorylation and intracellular accumulation of tau.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, Labour and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (Permit No. 22-23 and 11-028). All experiments were performed under sodium pentobarbital or isoflurane anesthesia, and every effort was made to minimize suffering.

Animals

P301L tau transgenic mice (JNPL3) (29) were purchased from Taconic (Hudson, NY) via IBL (Gunma, Japan). Granulin (GRN)-deficient (knockout [KO]) mice were obtained from RIKEN Bioresource Center (Tsukuba, Japan), which was established by Kayasuga et al (30). GRN-KO mice had been backcrossed to C57BL/6 J mice for more than 10 generations. Male P301L tau homozygote transgenic mice were interbred with female GRN-KO mice, or female P301L tau homozygote transgenic mice were interbred with male GRN-KO mice. P301L tau transgenic mice harboring the GRN hemizygote (Tau/GRN(+/-)) were produced. Control mice (Tau hemizygote) were produced by interbreeding male P301L tau homozygote transgenic mice and female C57BL/6 J. We used male mice in this study. The mice were reared in the animal facility of Tokyo Metropolitan Institute of Medical Science under conventional conditions at 24°C ± 2°C and were maintained on a commercial diet (CE-2; Nihon CLEA, Shizuoka, Japan) ad libitum.

Mice were killed under quick anesthesia with isoflurane (Mylan Pharmaceutical Company, Tokyo, Japan), and the brains were quickly removed. Brains of each group were cut in the sagittal plane; the left hemispheres were frozen and stored at −80°C for biochemical analyses. The right hemispheres were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 48 hours in the cold. Brain blocks were then transferred to a maintenance solution of 20% sucrose in 0.01 mol/L PBS, pH 7.4.

Sequential Fractionation of Brain Extracts

Frozen left hemispheres (~0.2 g) were homogenized in 10 volumes of buffer H (10 mmol/L Tris-HCl, pH 7.5; 0.8 mol/L NaCl; 1 mmol/L ethylene glycol bis-N,N,N',N'-tetraacetic acid; 1 mmol/L dithiothreitol). The hemisphere included the olfactory bulb, cerebral cortex, striatum, thalamus, hypothalamus, cerebellum, midbrain, pons, medulla oblongata, and the upper part of the spinal cord. The method used for sequential fractionation of brain extracts was originally described by Greenberg and Davies (31). Briefly, each brain homogenate was centrifuged at 100,000 × g for 20 minutes at 4°C; the supernatant was then collected as the Tris-soluble fraction. The resultant pellet was homogenized in 10 volumes of buffer H, followed by incubation for 30 minutes at 37°C with 1% Triton X-100. The homogenate was then centrifuged at 100,000 × g for 20 minutes at 4°C. The Triton X-100 insoluble pellet was sonicated in 5 volumes of buffer H, followed by incubation for 30 minutes at 37°C with 1% sarkosyl and centrifuged at 100,000 × g for 20 minutes at 4°C. The pellet was sonicated in 1 volume of

<table>
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<th>TABLE 1. Sources and Dilutions of Primary Antibodies</th>
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<tr>
<td><strong>Antibody</strong></td>
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<tr>
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<td>HT7</td>
</tr>
<tr>
<td>T46</td>
</tr>
<tr>
<td>pS422</td>
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<tr>
<td>phosphor-Akt substrate</td>
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<tr>
<td>phosphor-(Ser) CDKs substrate</td>
</tr>
<tr>
<td>phosphor-(Thr) MAPK/CDK substrate</td>
</tr>
<tr>
<td>phosphor-PKA substrate</td>
</tr>
<tr>
<td>phosphor-(Ser) PKC substrate</td>
</tr>
</tbody>
</table>

mAb, monoclonal antibody; pAb, polyclonal antibody.
sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis sample buffer.

**Immunoblotting Analysis**

For immunoblotting, brain extracts from the mice were boiled for 5 minutes with SDS–polyacrylamide gel electrophoresis sample buffer (60 mmol/L Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.025% bromophenol blue, and 5% mercaptoethanol) and loaded onto a 10% acrylamide minigel. Loaded samples were electrophoresed for 45 minutes at 200 V with molecular weight markers (Bio-Rad, Hercules, CA). Electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) for 60 minutes at 200 mA. The printed membranes were blocked with 3% gelatin for 30 minutes and then incubated in a primary antibody solution (T46, 1:3000; AT8, 1:2000; anti-pT181,

**FIGURE 1.** Total tau and phosphorylated tau of sequential fractionation protein samples. (A) Immunoblotting analysis was visualized using the T46 antibody for detecting total tau in the Tris-soluble fraction of the brains of 13-month-old mice. Four P301L tau mice and four P301L tau/GRN+/− mice were used for this assay. (B) A comparison of relative total tau expression levels in the Tris-soluble fraction of 13-month-old mice. The data were compared with the T46 band median intensity/α-tubulin. There was no significant difference between the 2 groups by Student t-test. (C) Immunoblotting analysis was visualized using AT8 antibody for detecting phosphorylated tau in the Tris-soluble fraction. Molecular weight markers are shown on the left (kDa). (D) A comparison of relative phosphorylated tau (AT8) expression levels in the Tris-soluble fraction of 13-month-old mice. The data were compared with the AT8 band median intensity/α-tubulin. $p < 0.05$ was considered significant by Mann-Whitney U test. a.u., arbitrary units.
1:2000; anti-pS422, 1:1000; or anti-α-tubulin, 1:10,000) (Table 1) overnight at room temperature. For phosphor-(Ser/Thr) kinase substrate assay, phospho-(Thr) mitogen-activated protein kinase/cyclin-dependent kinase (MAPK/CDK) substrate mouse monoclonal antibody (1:1000), phosphor-Akt substrate rabbit monoclonal antibody (1:1000), phosphor–protein kinase A (PKA) substrate rabbit monoclonal antibody (1:1000), phosphor–(Ser) protein kinase C (PKC) substrate rabbit polyclonal antibody (1:1000), and phosphor–(Ser) CDKs substrate rabbit polyclonal antibody (1:1000), which were obtained from Cell Signaling Technology, Inc (Danvers, MA), were used (Table 1). After incubation with a secondary antibody (1:50,000; Bio-Rad), immunoreactivity was detected by the chemiluminescence method using an ECL plus Western Blotting Detection Kit (GE Healthcare UK Ltd, Buckinghamshire, UK) or Super Signal West Dura (Thermo Scientific, West Palm Beach, FL) and was visualized with LAS-4000 mini (GE Healthcare UK Ltd). For quantitative measure of band intensity, α-tubulin was used as an internal control for protein concentration.

### Analysis of Tau Deposition

For fluorescent immunohistochemistry, sagittal sections were cut serially on a freezing microtome at 30-μm thickness, collected in the maintenance solution, and immunostained as free-floating sections. Sections were incubated for 24 hours with anti-pS422 antibody (1:500; Dr Hasegawa). The antibody labeling was visualized by incubation with Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:100; Invitrogen, Carlsbad, CA) for 3 hours. The sections were then rinsed with distilled water, mounted on glass slides, coverslipped with ProLong Gold Antifade Reagent (Invitrogen). Photographs were taken with a BioZero (Keyence, Osaka, Japan). The fluorescent positive cells were counted in the area of 820 × 1,080 μm of the external cortex of the inferior colliculus.

### Statistical Analyses

Data are presented as mean ± SE. The statistical significance of differences in the mean values between 2 populations was assessed with the Student t-test, whether

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**FIGURE 2.** Phosphorylated tau in the Tris-soluble fractions from the brains of 13-month-old mice. (A) Immunoblotting analysis was visualized using pT181 and pS422 and antibodies for detecting phosphorylated tau in Tris-soluble fraction of 13-month-old mice. Black arrowhead indicates phosphorylated tau. (B, C) A comparison of relative phosphorylated tau (pT181, pS422) expression levels. Data were compared with the band median intensity/α-tubulin. p < 0.05 was considered significant by Student t-test (B) and Mann-Whitney U test (C). a.u., arbitrary units. The loading control (α-tubulin) is also shown. Molecular weight markers are shown on the right (kDa).
variances were equal was determined by an F-test, and otherwise we used the Mann-Whitney U test. \( p < 0.05 \) was considered significant.

RESULTS

Phosphorylated Tau in the Tris-Soluble Fraction of 13-Month-Old Tau/GRN\(^{+/–}\) Mice

Brains were collected from 13-month-old mice of P301L tau or P301L tau/GRN\(^{+/–}\), and sequential protein extraction and immunoblotting were performed. On immunoblotting, total tau was detected by phosphorylation-independent anti-tau antibody, T46 (Fig. 1A, B); phosphorylated tau was revealed by phosphorylation-dependent anti-tau antibodies, including AT8 (Fig. 1C, D), pT181, and pS422 (Fig. 2A–C). The amount of total tau in the Tris-soluble fraction was almost the same in both mouse strains (Fig. 1A, B). On the other hand, the amounts of phosphorylated tau in the Tris-soluble fractions detected by AT8 (Fig. 1C, D), pT181 (Fig. 2A, B), and pS422 (Fig. 2A, C) were significantly higher in P301L tau/GRN\(^{+/–}\) mice than in P301L tau mice. The AT8/\( \alpha \)-tubulin ratio was 0.09 ± 0.06 (\( n = 4 \)) in the P301L tau mice and 1.02 ± 0.25 (\( n = 4 \)) in the P301L tau/GRN\(^{+/–}\) mice (Fig. 1D). The Mann-Whitney U test was used for statistical analysis, and there was a significant difference between the P301L tau mice and the P301L tau/GRN\(^{+/–}\) mice (\( F = 0.047, p = 0.03 \)). The pT181/\( \alpha \)-tubulin ratio was 0.16 ± 0.10 (\( n = 4 \)) in the P301L tau mice and 0.90 ± 0.25 (\( n = 4 \)) in the P301L tau/GRN\(^{+/–}\) mice (Fig. 2B). There was a significant difference between the P301L tau mice and the P301L tau/GRN\(^{+/–}\) mice (\( F = 0.017, p = 0.04 \)). The pS422/\( \alpha \)-tubulin ratio was 0.20 ± 0.06 (\( n = 4 \)) in the P301L tau mice and 1.73 ± 0.37 (\( n = 4 \)) in the P301L tau/GRN\(^{+/–}\) mice (Fig. 2C). There was a significant difference between the P301L tau mice and the P301L tau/GRN\(^{+/–}\) mice (\( F = 0.016, p = 0.02 \)). Phosphorylated tau was not detected in the sarkosyl-insoluble fraction in either mouse strain (data not shown).

Phosphorylated Tau in the Sarkosyl-Insoluble Fraction of 19-Month-Old P301L Tau/GRN\(^{+/–}\) Mice

Brains were collected from 19-month-old mice of P301L tau or P301L tau/GRN\(^{+/–}\), and sequential protein extraction and immunoblotting were performed. Phosphorylated tau in the sarkosyl-insoluble fraction was visualized by

FIGURE 3. Immunoblotting analysis of phosphorylated tau in sarkosyl-insoluble fractions from the brains of 19-month-old mice. (A) Immunoblotting analysis was visualized using the AT8 antibody for detecting phosphorylated tau in sarkosyl-insoluble fractions from brains of 19-month-old mice. Black arrowhead indicates phosphorylated tau. Molecular weight markers are shown on the right (kDa). (B) A comparison of relative phosphorylated tau (AT8) expression levels in the sarkosyl-insoluble fractions. Data were compared with the AT8 band median intensity. \( p < 0.05 \) was considered to significant by Mann-Whitney U test. a.u., arbitrary units.
Western blotting using the AT8 antibody (Fig. 3A). The median intensities were significantly higher in the P301L tau/GRN<sup>−/−</sup> mice (20,156.3 ± 1,296.5, n = 8) than in the P301L tau mice (14,720.4 ± 470.1, n = 8) (Fig. 3B). These results suggest that the level of abnormally phosphorylated tau accumulation was increased in the P301L tau/GRN<sup>−/−</sup> mice as compared with the P301L tau mice.

**Phosphorylated Tau in 19-Month-Old Mice by Immunofluorescence Staining**

Phosphorylated tau in the area of the external cortex of the inferior colliculus of 19-month-old P301L tau and P301L tau/GRN<sup>+</sup>/<sup>+</sup> mice was visualized using immunofluorescence staining with an anti-pS422 antibody (Fig. 4). The pS422-positive cells were increased in P301L tau/GRN<sup>−/−</sup> mice (289 ± 43 cells, n = 3) compared with P301L tau mice (94 ± 23 cells, n = 3) (F = 3.528, p = 0.03 by Student t-test).

**Kinase Activity Using Phosphor-(Ser/Thr) Kinase Substrate Antibodies**

Determining kinase activities in the brains of P301L tau and P301L tau/GRN<sup>+</sup>/<sup>+</sup> mice by immunoblotting was performed using phosphor-(Ser/Thr) kinase substrate antibodies. There were no differences detected for phospho-(Thr) MAPK/CDK substrate, phospho-Akt substrate, phospho-PKA substrate, and phospho-(Ser) PKC substrate in Tris-soluble fractions of 13-month-old P301L tau and P301L tau/GRN<sup>−/−</sup> mice (Fig. 5A–D). By contrast, the phospho-(Ser) CDK substrate was more prominently phosphorylated in P301L tau/GRN<sup>−/−</sup> mice than in the P301L tau mice (Fig. 5E). The approximately 60-kDa band/α-tubulin ratio was 2.04 ± 0.22 (n = 4) in the P301L tau mice and 3.08 ± 0.39 (n = 4) in the P301L tau/GRN<sup>−/−</sup> mice (Figure, part A, Supplemental Digital Content 1, http://links.lww.com/NEN/A697). There was a significant difference between the P301L tau mice and the P301L tau/GRN<sup>−/−</sup> mice (F = 0.358, p = 0.03; Figure, part B, Supplemental Digital Content 1, http://links.lww.com/NEN/A697) (Student t-test).

**DISCUSSION**

The results of the present study show that GRN mutations causing PGRN reduction are associated with increased phosphorylation and intracellular accumulation of tau in mice harboring the P301L tau mutation. They suggest that GRN mutations causing PGRN reduction may be causative or risk factors for tauopathies. Indeed, there are many reports of GRN mutations associated with AD (Table 2). Furthermore, in a large Belgian FTLD pedigree with a GRN ISV1+5G > C mutation, a few patients had a cerebrospinal fluid biomarker profile typical of AD, that is, decreased amyloid-β protein (1–42) and increased total tau and phosphorylated tau (p181) (12). Another patient in the pedigree had comorbidity of FTLD-TDP and dementia with Lewy bodies with AD pathology at autopsy.

In this study, we showed accelerated phosphorylation of tau in Tris-soluble and sarkosyl-insoluble fractions in P301L tau/GRN<sup>−/−</sup> mice compared with P301L tau mice. To identify the responsible kinases, we performed immunoblotting analysis using phosphor-(Ser/Thr) kinase substrate antibodies.

**FIGURE 4.** Immunofluorescence staining of abnormal tau. (A, B) pS422 immunoreactivity was observed in the midbrain of P301L tau mice (A) and P301L tau/GRN<sup>−/−</sup> mice (B). The calibration bar in (A) applies to both panels (200 μm). (C) Comparison of pS422-positive cell numbers in the 2 sets of mice. The data were compared with the pS422-positive cell numbers in the external cortex of the inferior colliculus. p < 0.05 was considered significant by Student t-test.
Strong phosphorylation of CDK substrates observed in P301L tau/GRN+/- mice brain compared with P301L tau mice suggests that CDKs might be activated by GRN deficiency resulting in increased phosphorylated tau (Fig. 5E). There were no significant differences of phosphorylation levels in substrates for MAPK, Akt, PKA, or PKC (Fig. 5A-D). Furthermore, there were no significant differences in the amount of the phosphorylated GSK-3a in the Tris-soluble fraction between P301L tau/GRN+/- mice and P301L tau mice (data not shown). These results suggest that these kinases were not involved in the accelerated phosphorylation of tau in P301L tau/GRN+/- mice.

The mechanisms by which GRN hemi-knockout may activate CDKs and accelerate tau pathology in P301L tau mice are not known and require future exploration. The proinflammatory effects of PGRN downregulation might also accelerate abnormal tau phosphorylation. There is a report

**FIGURE 5.** Kinase activities in the brains of 13-month-old P301L tau/GRN+/- and P301L tau mice. (A–E) Kinase activities in the brains were visualized using phosphor-(Ser/Thr) kinase substrate antibodies as follows: phosphor-MAPK, mitogen-activated protein kinase (p-MAPK) substrate antibody (A), phosphor-Akt substrate antibody (p-AKT) (B), phosphor-protein kinase A (p-PKA) substrate antibody (C), phosphor-protein kinase C (p-PKC) substrate antibody (D), and p-cyclin-dependent kinases (CDKs) substrate antibody (E). Molecular weight markers are shown on the right (kDa).

**TABLE 2.** GRN Mutations That Have Been Confirmed in Patients Clinically Diagnosed as Having Alzheimer Disease

<table>
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<tr>
<th>Putative Loss-of-Function Mechanism</th>
<th>Mutation (Gene)</th>
<th>Mutation (Protein)</th>
<th>Clinical Phenotype</th>
<th>Reference</th>
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<td>Loss of transcript</td>
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AD, Alzheimer disease; CBS, corticobasal syndrome; FTD, frontotemporal dementia; FTLD, frontotemporal lobar degeneration; PA, progressive nonfluent aphasia; PD, Parkinson disease; p.0, progranulin null.
that PGRN binds to tumor necrosis factor receptors and prevents inflammation (32). Recently, a number of studies with tau transgenic mice have shown that inflammation exacerbates tau pathology in these mice (33–35). In the present study, we measured expression levels of inflammatory cytokines (interleukin 1β) or cyclooxygenase-2, but they were not increased in P301L tau/GRN+/- mice (data not shown).

The mechanism of CDK activation and accumulation of phosphorylated tau caused by PRGN deficiency should be investigated further, but the results of the present study suggest that GRN mutations might contribute to the pathogenesis or be risk factors for tauopathies, including AD. Controlling PGRN levels or suppressing CDK activation may be useful therapy in tauopathy cases with GRN mutations.

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