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## Reducing inflammation and rescuing FTD-related behavioral deficits in progranulin-deficient mice with $\alpha 7$ nicotinic acetylcholine receptor agonists

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### ABSTRACT

Mutations in the progranulin gene cause frontotemporal dementia (FTD), a debilitating neurodegenerative disease that involves atrophy of the frontal and temporal lobes and affects personality, behavior, and language. Progranulin-deficient mouse models of FTD exhibit deficits in compulsive and social behaviors reminiscent of patients with FTD, and develop excessive microgliosis and increased release of inflammatory cytokines. Activation of nicotinic acetylcholine receptors (nAChRs) by nicotine or specific  $\alpha 7$  nAChR agonists reduces neuroinflammation. Here, we investigated whether activation of nAChRs by nicotine or  $\alpha 7$  agonists improved the excessive inflammatory and behavioral phenotypes of a progranulin-deficient FTD mouse model. We found that treatment with selective  $\alpha 7$  agonists, PHA-568487 or ABT-107, strongly suppressed the activation of NF- $\kappa$ B in progranulin-deficient cells. Treatment with ABT-107 also reduced microgliosis, decreased TNF $\alpha$  levels, and reduced compulsive behavior in progranulin-deficient mice. Collectively, these data suggest that targeting activation of the  $\alpha 7$  nAChR pathway may be beneficial in decreasing neuroinflammation and reversing some of the behavioral deficits observed in progranulin-deficient FTD.

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### 1. Introduction

Frontotemporal dementia (FTD) is a clinical presentation that encompasses a broad group of disorders that result in atrophy of the frontal and temporal lobes of the brain—areas associated with personality, behavior, and language. Mutations in the progranulin gene (*GRN*), which cause haploinsufficiency of progranulin levels, are a major cause of FTD [1–3]. Progranulin-deficient mice develop excessive microgliosis and secrete increased levels of inflammatory cytokines, key elements of neuroinflammation [4–6]. Preclinical

studies suggest a compelling role for the nicotinic cholinergic system in reducing inflammation in the brain, implicating it as a potential therapeutic target for alleviating progranulin deficiency-associated deficits in FTD.

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated channels composed of a pentameric complex of a possible 12 different subunits. In the brain, nAChRs are widely distributed in both neurons and glia, and predominantly comprise the  $\alpha 7$  and  $\alpha 4\beta 2$  subtypes [7,8]. In neurons, nAChRs are involved in a number of physiological functions.  $\alpha 7$ -containing nAChRs in particular are highly permeable to calcium, implicating them as significant modulators of intracellular signaling and neurotransmitter release and, consequently, in the pathophysiology of a variety of neurological diseases. Indeed, loss of basal forebrain cholinergic neurons and decreased production of ACh significantly contributes to early Alzheimer's disease dementia. In animal models, nicotine enhances long-term potentiation [9] and episodic and working memory [10]. Conversely, anti- $\alpha 7$  nAChR antibodies induced inflammation and increased amyloid accumulation in mouse models of Alzheimer's disease [11]. Recent studies have

**Abbreviations:** nAChR, nicotinic acetylcholine receptor; FTD, frontotemporal dementia; PGRN, progranulin; LPS, lipopolysaccharide; PFA, paraformaldehyde; IP, intraperitoneal; DAB, 3,3'-diaminobenzidine; GM-CSF, granulocyte macrophage colony-stimulating factor.

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demonstrated a protective effect of  $\alpha 7$  nAChRs in decreasing L-Dopa-induced dyskinesias in Parkinson's disease as well, implicating an emerging role for  $\alpha 7$  nAChRs in multiple therapeutic areas [12].

nAChRs have also been implicated in the cholinergic anti-inflammatory pathway, as they are also expressed in non-neuronal cells of the brain. The  $\alpha 7$  subunit-containing receptors in particular modulate innate immunity and inflammatory responses by regulating the release of inflammatory cytokines and chemokines [13,14]. Administration of  $\alpha 7$  nAChR agonists inhibited release of TNF $\alpha$ , IL-1, IL-6, and IL-8 [15]. Furthermore, activation of  $\alpha 7$  nAChRs resulted in decreased translocation of NF- $\kappa$ B to the nucleus [15], a critical event in triggering downstream inflammatory pathways.

Thus, activating nAChRs, especially  $\alpha 7$  subtypes, may attenuate the increased microgliosis and inflammatory cytokine release observed in progranulin-deficient mice. In the current study, we aimed to determine whether nicotine, or specific  $\alpha 7$  agonists of nAChRs, could indeed reverse the excessive neuroinflammation and behavioral deficits observed in a mouse model of progranulin-deficient FTD.

## 2. Materials and methods

### 2.1. Mice

For all experiments, male and female mice were used in gender-balanced groups. *Grn*<sup>-/-</sup> mice were obtained from the laboratory of Robert V. Farese, Jr [16]. All mice were housed in a pathogen-free barrier facility with a 12-h light/dark cycle and *ad libitum* access to food and water. All behavior experiments were conducted during daylight hours unless otherwise noted. All animal procedures were carried out under University of California, San Francisco, Institutional Animal Care and Use Committee-approved guidelines.

### 2.2. Chemicals

LPS and nicotine were purchased from Sigma (St. Louis, MO). PHA-568487 and recombinant TNF $\alpha$  were purchased from R&D Systems (Minneapolis, MN). ABT-107, a full  $\alpha 7$  agonist 5-(6-[(3R)-1-azabicyclo[2.2.2]oct-3-yloxy]pyridazin-3-yl)-1H-indole, was obtained from AbbVie Inc. (North Chicago, IL).

### 2.3. Generation of Bone Marrow-Derived Macrophages and Microglia for NF- $\kappa$ B Reporter Assay

Bone marrow-derived cells were obtained from 2-month-old *Grn*<sup>-/-</sup> mice. To immortalize myeloid cells, Ficoll-purified progenitors were infected with ER-Hoxb8 retrovirus and maintained in granulocyte macrophage colony-stimulating factor (GM-CSF) medium with estrogen as described [17]. Cells were transduced with pGreenFire-5 $\kappa$ B lentivirus expressing both GFP and luciferase as reporters to generate immortalized myeloid cell lines. For experiments, macrophages were differentiated from ER-Hoxb8 GM-CSF myeloid progenitor cells by plating in medium without estrogen. For primary microglia experiments, cortices from postnatal day 3 *Grn*<sup>+/+</sup> or *Grn*<sup>-/-</sup> mice were isolated and plated in T75 flasks in DMEM, 10% FBS, GlutaMAX, 2% B16-conditioned medium, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. At DIV 7, flasks were shaken for 2 h and detached microglia were replated in 96-well plates in B16-conditioned medium-free medium. Microglia were treated with Lenti-pGreenFire-5 $\kappa$ B on the same day, and experiment was conducted 48 h following infection. Myeloid or microglia were pre-treated with nicotine or PHA for indicated time, followed by treatment with recombinant TNF $\alpha$  or LPS for 2 h. Cells were lysed in luciferase assay buffer

(Promega, Madison, WI), and luciferase activity was read on a Victor luminometer (PerkinElmer, Waltham, MA).

### 2.4. Drug Dosing

In all experiments, mice were treated for 14 days by either Alzet osmotic mini-pumps or daily intraperitoneal (I.P.) injection. For Alzet mini-pump experiments, mice received a total per day of 4.2 mg/kg nicotine, 0.42 mg/kg PHA-568487, or 0.45 mg/kg ABT-107 at a rate of 0.25  $\mu$ l/h. Osmotic pumps were implanted subcutaneously under the skin on the dorsum while mice were anesthetized with Avertin (Sigma). For I.P. experiments, mice received a once daily injection of 0.6 mg/kg nicotine or 0.064 mg/kg ABT-107. For both I.P. and osmotic pump experiments, nicotine and PHA-568487 were solubilized in saline, and ABT-107 was solubilized with 10 N HCl, followed by further dilution with water.

### 2.5. Brain Harvest and Quantification of Microglia

Mice were transcardially perfused with 0.9% saline after deep anesthesia with Avertin. One hemi-brain was fixed with 4% PFA for 24 h, incubated in 30% sucrose for 24 h, and sectioned on a Leica freezing microtome (30  $\mu$ m-thick sections). The other hemi-brain was snap-frozen in dry ice for mRNA analyses. Experimenters were blind to the genotypes or treatment of the mice for all immunohistochemical analyses. Sections were washed and incubated with anti-CD68 (MCA1957GA, 1:500, Serotec, Raleigh, NC) after quenching endogenous peroxidase activity. Staining of CD68 was detected with 0.25 mg/ml DAB with 0.01% H<sub>2</sub>O<sub>2</sub>. Images were acquired on a brightfield Leica DM5000B microscope with a Leica DFC310 FX camera and staining was quantified using NIH ImageJ software.

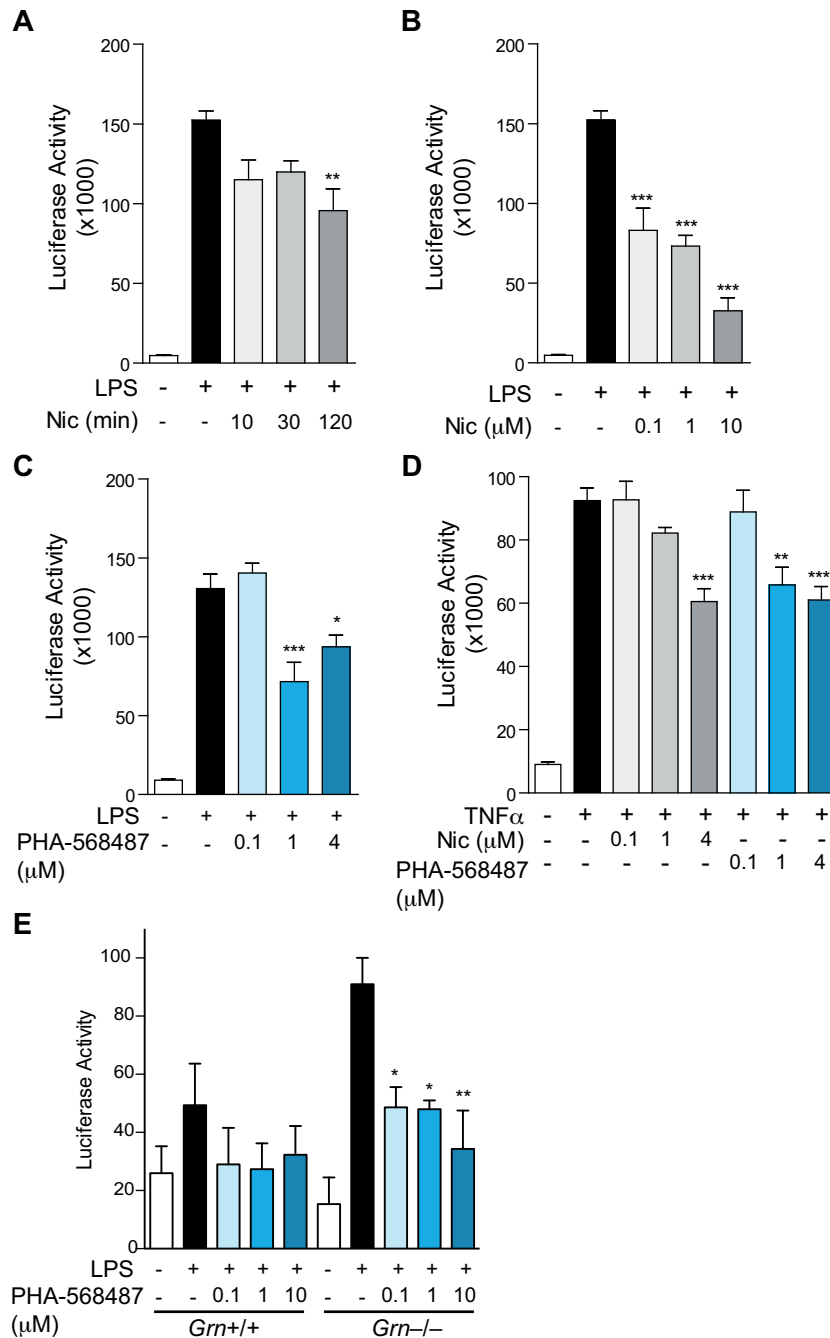
For immunofluorescence staining, sections were incubated with anti-Iba1 (#019-19741, 1:500, Wako, Osaka, Japan) after permeabilization in Tris-buffered saline with 0.5% Triton X-100 (TBST) and blocking with 10% normal donkey serum. Immunoreactive structures were detected with Alexa Fluor donkey anti-rabbit 488 secondary antibody (Invitrogen, Carlsbad, CA). Images were acquired on a Leica DM5000B microscope and quantified with Volocity software (PerkinElmer, Waltham, MA). All DAB and fluorescent imaging and quantification were performed in the cortical region of brains.

### 2.6. RNA Isolation and Quantitative Real-Time PCR

To measure mRNA of cytokines, cortical brain tissue was homogenized with a 21G needle in RLT buffer with 1%  $\beta$ -ME. RNA was isolated per the RNeasy isolation kit instructions (Qiagen, Valencia, CA), and the remaining DNA in the sample was removed by incubation with RNase-free DNase (Ambion, Austin, TX). RNA was then converted to cDNA by the TaqMan reverse transcription (RT) kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed on the ABI 7900HT sequence detector (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems). The relative gene expression was quantified as  $2^{-\Delta C_t}$ . The mean value of replicates of each sample was expressed as the threshold cycle ( $C_t$ ) at which the level of fluorescence begins to increase rapidly. The amount of gene expression was calculated as the difference ( $\Delta C_t$ ) between the  $C_t$  value of the sample for target gene and the  $C_t$  value of that sample for the endogenous control GAPDH ( $\Delta C_t = C_t(\text{target gene}) - C_t(\text{GAPDH})$ ). The relative amount of gene expression for each target gene was determined by  $2^{-\Delta C_t}$  and expressed as the fold change as compared with a control. The primers used were as follows: GAPDH sense GGG AAG CCC ATC ACC ATC TT, antisense GCC TTC TCC ATG GTG GTG AA; TNF $\alpha$  sense CAT CAG TTC TAT GGC CCA GA, antisense TGC TCC TCC ACT TGG TGG TT;

IL-1 $\alpha$  sense CAC CTT ACA CCT ACC AGA GTG ATT TG, antisense TGT TGC AGG TCA TTT AAC CAA GTG; IL-1 $\beta$  sense TGA AAG ACG GCA CAC CCA CCC, antisense TGC CCT GGG GAA GGC ATT AGA; IL6 sense ACC ACT TCA CAA GTC GGA GGC T, antisense TCT CTG AAG GAC TCT GGC TTT GTC T; TGF $\beta$  sense AAC CCC CAT TGC TGT CCC GTG,

antisense GCG CTG AAT CGA AAG CCC TGT; IL4 sense TGC GAA GCA CCT TGG AAG CCC, antisense GGG ACG CCA TGC ACG GAG ATG; CD11b sense GTG TGA CTA CAG CAC AAG CCG, antisense CCC AAG GAC ATA TTC ACA GCC T.



**Fig. 1.** Nicotine inhibits NF- $\kappa$ B activation. (A) Increasing time of pre-treatment with 4  $\mu$ M nicotine decreased the luciferase signal from progranulin knock-out myeloid cells expressing pGreenFire-5 $\kappa$ B reporter construct and treated with 10 ng/ml LPS for 2 h.  $n = 5-6$  wells per condition.  $**P < 0.01$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (B) Dose-dependent decrease of luciferase signal in response to nicotine in progranulin knock-out myeloid cells expressing pGreenFire-5 $\kappa$ B reporter construct and treated with 10 ng/ml LPS for 2 h.  $n = 6$  wells per condition.  $***P < 0.001$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (C) Pre-treatment with increasing concentrations of PHA-568487 decreased the luciferase signal from progranulin knock-out myeloid cells expressing pGreenFire-5 $\kappa$ B reporter construct and treated with 10 ng/ml LPS for 2 h.  $n = 4$  wells per group.  $*P < 0.05$ ,  $***P < 0.001$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (D) Pre-treatment with increasing concentrations of nicotine or PHA-568487 decreased, in a dose-dependent manner, the luciferase signal from progranulin knock-out myeloid cells expressing pGreenFire-5 $\kappa$ B reporter construct and treated with 100 ng/ml TNF $\alpha$  for 2 h.  $n = 4$  wells per group.  $**P < 0.01$ ,  $***P < 0.001$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (E) Pre-treatment with increasing concentrations of PHA-568487 decreased, in a dose-dependent manner, the luciferase signal from progranulin knock-out microglia infected with pGreenFire-5 $\kappa$ B reporter construct and treated with 10 ng/ml LPS for 2 h.  $n = 3$  wells per group.  $*P < 0.05$ ,  $**P < 0.01$  by ANOVA followed by Tukey-Kramer *post-hoc* test.

## 2.7. Behavior tests

## 2.7.1. Three-Chambered Social Test

For the habituation phase, mice were allowed to freely explore a three-chambered box including two empty wire cups in the outer chambers for 10 min under dim light. Mice were then closed into the center chamber, and a conspecific stranger mouse was placed under one wire cup and a green Lego toy was placed under the other wire cup. Barriers were released, and the test mice were allowed to freely explore the box, now including a stranger mouse and an object, for 10 min. In both phases, the amount of time spent sniffing (interaction time) was measured for the mouse cup and the object cup. Data were presented as ratio of sniffing time for the mouse cup over sniffing time for the object cup. The boxes were thoroughly cleaned with 70% ethanol between mouse sessions.

## 2.7.2. Elevated Plus Maze

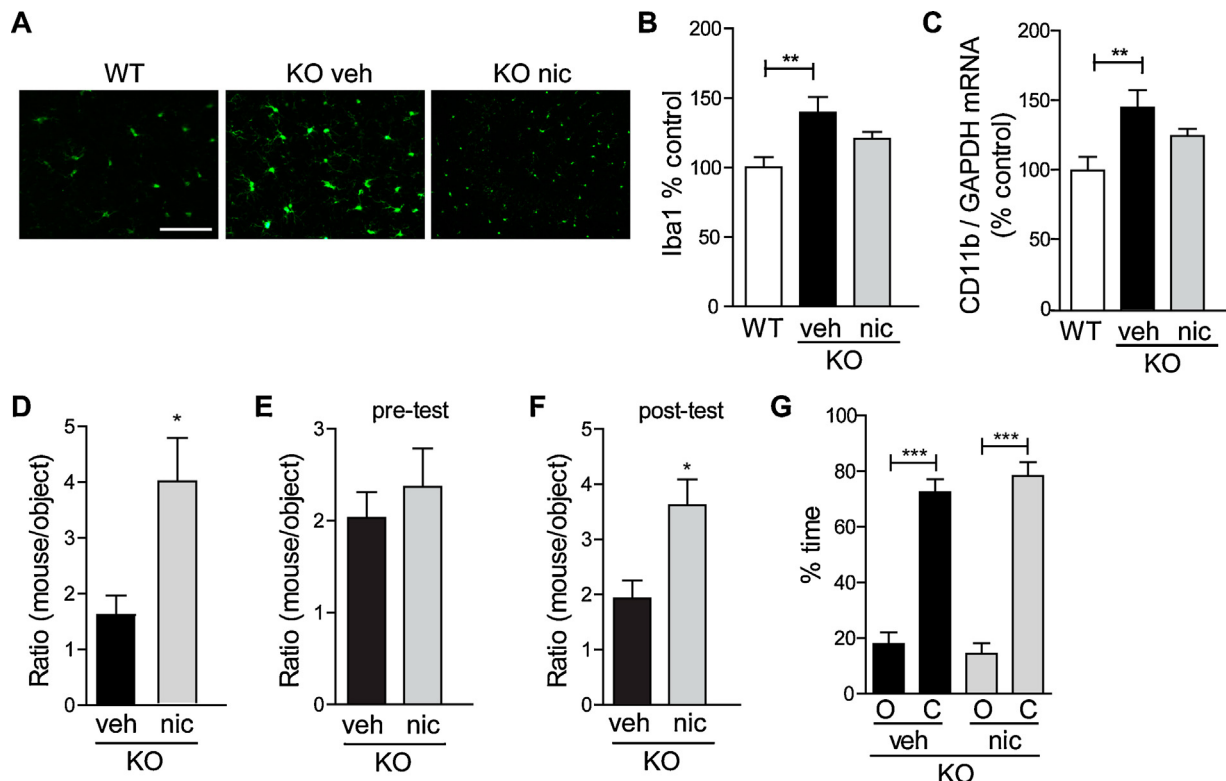
The elevated plus maze consisted of two open (without walls) and two enclosed (with walls) arms elevated 63 cm above the ground (Hamilton-Kinder, Poway, CA). Mice were first allowed to habituate in the testing room under dim light for 30 min before testing. During testing, mice were placed at the junction between the open and closed arms of the plus maze and allowed to explore for 10 min. The distance traveled and the time spent in the open and closed arms were determined. The maze was thoroughly cleaned with 70% ethanol between testing sessions.

## 2.7.3. Burrowing Test

All burrowing tests were performed at the same time of the day to ensure consistency [18]. Briefly, at 6:00 pm, mice were placed in a clean cage with a burrow (plastic canister with one end open) containing 300 g of food pellets. The open end of the burrow was raised slightly above the cage floor. At 9:00am the next morning, the burrows were weighed, and the difference in weight was recorded as the amount of food burrowed.

## 2.8. Statistical Analysis

Data were analyzed with GraphPad Prism v.5 (GraphPad, San Diego, CA). Differences between means were assessed with paired Student's *t* test, or with one-way or two-way analysis of variance followed by *post hoc* testing of all pairwise comparisons among groups with Tukey-Kramer correction for multiple comparisons. Pearson's correlation coefficients were used to quantify the linear relationship between two variables. The Shapiro-Wilk test of normality was applied to all data sets, and in cases where the data did not demonstrate a normal distribution, non-parametric tests were used to analyze statistical differences. The Wilcoxon matched pairs test was used for paired comparisons. An F test or Bartlett's test was performed to determine significant differences in variances between groups for *t*-tests and ANOVAs, respectively. All values are given as mean  $\pm$  S.E.M.



**Fig. 2.** Nicotine reduces progranulin-deficiency-induced microgliosis and rescues social behavior deficits. (A–D, G) Six-month-old progranulin knock-out mice were injected daily (I.P.) with vehicle control or 0.6 mg/kg nicotine for 14 days. (A) Representative images of cryosections immunostained for Iba1-positive microglia from wild-type or progranulin knock-out mice injected with vehicle or nicotine. (B) Quantification of Iba1-positive immunoreactivity (right).  $n = 7–10$  mice per group.  $**P < 0.01$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (C) CD11b mRNA levels were measured by qRT-PCR from brains and normalized to GAPDH mRNA levels.  $n = 7–9$  mice per group.  $**P < 0.01$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (D) One day after the last injection, mice were tested in the three-chambered social test. The amount of time spent interacting with a stranger mouse was normalized to the amount of time spent interacting with an object, shown as a ratio of mouse/object.  $n = 6–7$  mice per group.  $*P < 0.05$  by student's *t*-test. (E–F) Twelve-month-old progranulin knock-out mice were injected daily (I.P.) with vehicle control or 0.6 mg/kg nicotine for 14 days. Mice were tested in the three-chambered social test before (E) and after (F) treatment.  $n = 6$  mice per group.  $*P < 0.05$  by student's *t*-test. (G) Six-month-old progranulin knock-out mice were tested in the elevated plus maze. Amount of time spent in the open arms (O) and closed arms (C) is shown.  $n = 7–8$  mice per group.  $***P < 0.001$  by paired *t*-test. Scale bar: 100  $\mu$ m.

### 3. Results

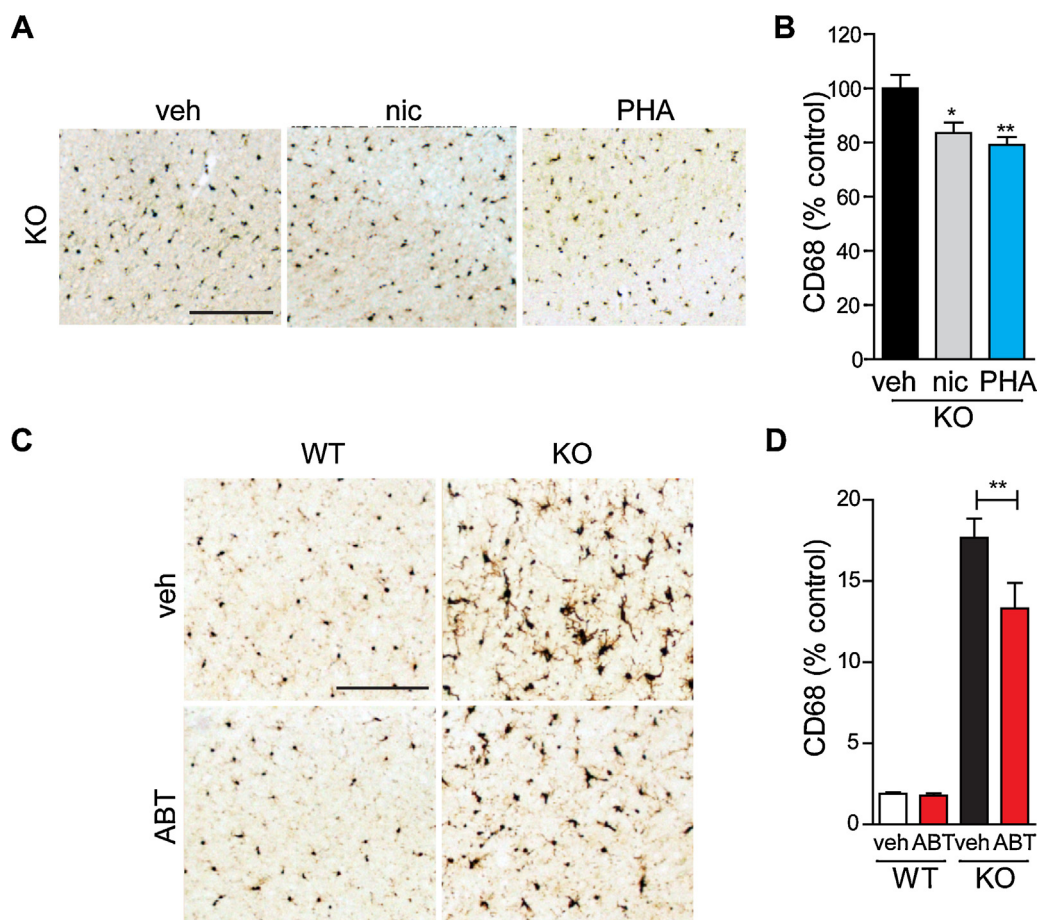
Upon activation, *Grn*<sup>-/-</sup> microglia are susceptible to a hyperactive proinflammatory state and release increased levels of proinflammatory cytokines than *Grn*<sup>+/+</sup> microglia [16]. NF- $\kappa$ B signaling is a master regulator of inflammatory mediators. To determine the effects of nicotine on NF- $\kappa$ B signaling in progranulin-deficient cells, we established an NF- $\kappa$ B reporter macrophage line by immortalizing bone marrow-derived macrophages stably expressing luciferase under 5 repeats of  $\kappa$ B enhancer element (5 $\times$  $\kappa$ B) that binds to NF- $\kappa$ B p65/p50 dimers [19]. Cells were treated with nicotine before stimulation with lipopolysaccharide (LPS). LPS treatment of *Grn*<sup>-/-</sup> cells markedly increased their NF- $\kappa$ B-dependent luciferase expression, which was significantly reduced by nicotine pretreatment in a dose-dependent manner (Fig. 1A, B).

Nicotine is a non-selective agonist of nAChRs. Since  $\alpha$ 7 nAChRs in particular have been implicated in the cholinergic anti-inflammatory pathway [20], we examined whether specific agonists of the  $\alpha$ 7 nAChR could attenuate LPS-induced NF- $\kappa$ B in *Grn*<sup>-/-</sup> macrophages. PHA-568487 was developed to activate  $\alpha$ 7 nAChR [21]. PHA-568487 (at 1 or 4  $\mu$ M) decreased NF- $\kappa$ B-dependent luciferase signal in response to LPS stimulation (Fig. 1C). We then induced NF- $\kappa$ B activation by TNF $\alpha$  signaling since excessive TNF $\alpha$  signaling has been implicated in GRN haploinsufficiency [22]. Increasing concentrations of either nicotine or PHA-

568487 resulted in a dose-dependent reduction of the TNF $\alpha$ -stimulated increase in NF- $\kappa$ B activation in *Grn*<sup>-/-</sup> macrophages (Fig. 1D). We then directly compared LPS-induced NF- $\kappa$ B activation in *Grn*<sup>+/+</sup> and *Grn*<sup>-/-</sup> microglia and their responses to PHA-568487. Microglia from *Grn*<sup>-/-</sup> mice exhibited significant NF- $\kappa$ B activation upon stimulation, as expected. The modest induction of NF- $\kappa$ B activation induced by LPS stimulation in wildtype microglia did not reach statistical significance, most likely due to low concentration of LPS. Notably, treatment with PHA-568487 led to a dose-dependent suppression of NF- $\kappa$ B activation in *Grn*<sup>-/-</sup> microglia (Fig. 1E).

*Grn*<sup>-/-</sup> mice exhibit elevated microgliosis in multiple brain regions, including cortex and thalamus [4,16,23]. To determine the *in vivo* effects of nicotine, we injected *Grn*<sup>-/-</sup> mice with 0.6 mg/kg of nicotine once a day, I.P., for 14 days (Fig. 2). Brain sections were immunostained for Iba1, a microglial marker. Vehicle-injected *Grn*<sup>-/-</sup> mice had significantly more Iba1 immunoreactivity than age-matched wild-type controls (Fig. 2A, B). Correspondingly, mRNA levels of CD11b, another marker for microglia, were increased in vehicle-injected *Grn*<sup>-/-</sup> mice (Fig. 2C). Injection of nicotine induced a trend of reduction in Iba1 immunoreactivity or levels of CD11b control, which did not reach statistical significance (Fig. 2B, C).

Decreased sociability is the primary behavioral deficit observed in *Grn*<sup>-/-</sup> mice [5]. Compared to wild-type controls, knock-out mice spend less time interacting with a conspecific mouse than with an



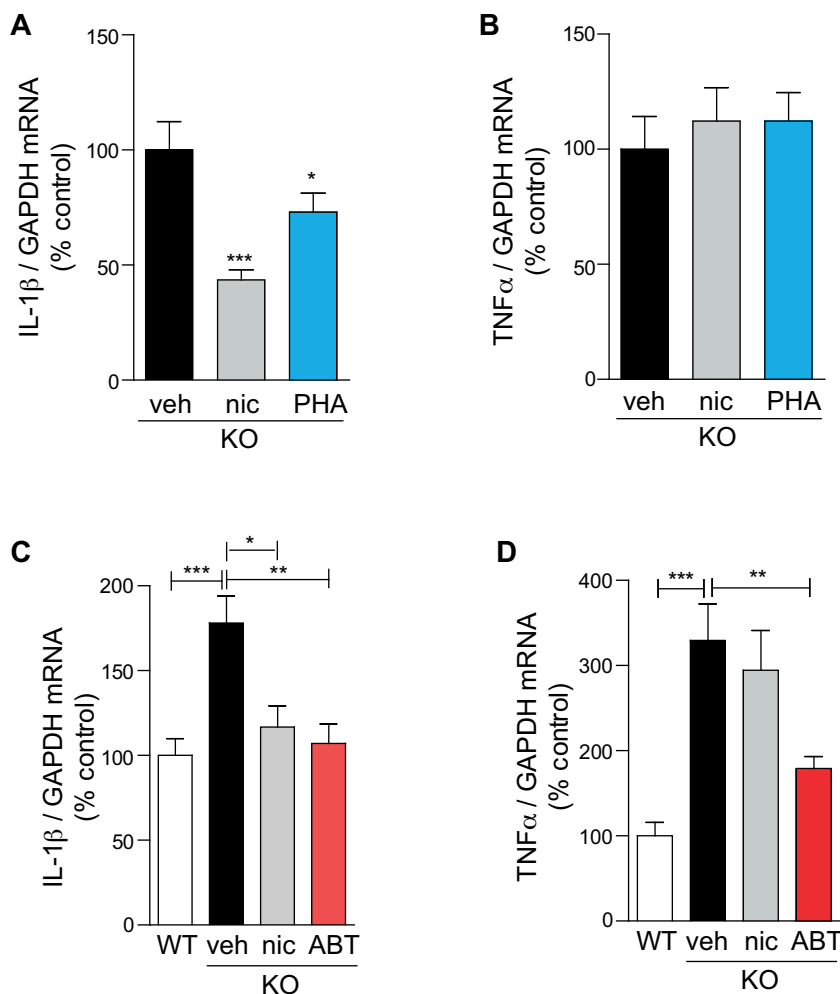
**Fig. 3.** Selective  $\alpha$ 7 nAChR agonists PHA-568487 and ABT-107 decrease microgliosis. (A–B) Nine-month-old progranulin knock-out mice were treated with vehicle, nicotine, or PHA-568487 by Alzet osmotic mini-pumps for 14 days. (A) Representative images of cortical cryosections immunostained for CD68-positive microglia from vehicle, nicotine, or PHA-568487 treated mice. (B) Quantification of CD68 immunoreactivity.  $n = 10$  mice per group. \* $P < 0.05$ , \*\* $P < 0.01$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (C–D) Sixteen-month-old wild-type or progranulin knock-out mice were injected daily (I.P.) with vehicle or ABT-107 for 14 days. (C) Representative images of cortical cryosections immunostained for CD68-positive microglia from wild-type or progranulin knock-out mice treated with vehicle or ABT-107. (D) Quantification of CD68 immunoreactivity.  $n = 5–7$  mice per group. \*\* $P < 0.01$  by two-way ANOVA followed by Bonferroni *post-hoc* test. Scale bars: 200  $\mu$ m.

object in the three-chamber social test [5]. *Grn*<sup>-/-</sup> mice injected with nicotine preferred to spend more time with the mouse than those injected with vehicle (Fig. 2D), suggesting beneficial effects of nicotine. Importantly, the sociability deficits of 12 month-old *Grn*<sup>-/-</sup> mice were also reversed with daily nicotine treatment for 14 days, suggesting beneficial effects of nicotine even when disease phenotype has already been established (Fig. 2E, F). Nicotine treatment did not seem to affect the degree of anxiety in *Grn*<sup>-/-</sup> mice. In the elevated plus maze, vehicle- and nicotine-injected *Grn*<sup>-/-</sup> mice spent a similar percentage of time in the open or closed arms (Fig. 2G).

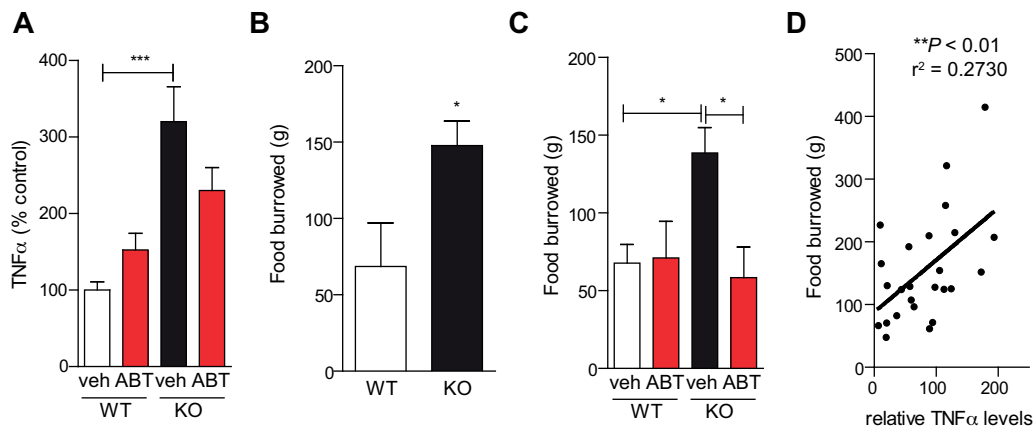
Next, we examined the anti-inflammatory effects of  $\alpha 7$  nAChR signaling by directly comparing nicotine and PHA-568487 in *Grn*<sup>-/-</sup> mice. We implanted Alzet osmotic mini-pumps in *Grn*<sup>-/-</sup> mice to infuse vehicle, nicotine, or PHA-568487 for 14 days. Additionally, we tested the efficacy of a second specific agonist of the  $\alpha 7$  nAChR, ABT-107, which has previously been shown to decrease secretion of inflammatory cytokines [24,25]. ABT-107 was administered daily for 14 days (I.P.). Compared with vehicle treatment, nicotine or PHA-568487 treatment significantly suppressed CD68 immunoreactivity, a marker for activated microglia (Fig. 3A, B). ABT-107 also significantly decreased CD68 immunoreactivity in *Grn*<sup>-/-</sup> mice (Fig. 3C, D).

To further confirm the importance of  $\alpha 7$  nAChRs in progranulin-deficiency-induced inflammation, we tested the efficacy of  $\alpha 7$  nAChR agonists on inflammatory cytokine expression. Nicotine or PHA-568487 treatment by Alzet osmotic pump significantly reduced levels of the pro-inflammatory cytokine IL-1 $\beta$  (Fig. 4A), but not TNF $\alpha$  (Fig. 4B). Infusion of ABT-107 and nicotine also induced a similar reduction in the levels of IL-1 $\beta$  (Fig. 4C). Interestingly, levels of TNF $\alpha$  were significantly reduced by ABT-107, but not nicotine, suggesting that ABT-107 may be a more potent inhibitor of excessive TNF $\alpha$  signaling in *Grn*<sup>-/-</sup> mice (Fig. 4D).

We further examined the *in vivo* effects of ABT-107 by treating wild-type or *Grn*<sup>-/-</sup> mice with vehicle or ABT-107 daily, I.P., for 14 days. ABT-107 treatment reduced TNF $\alpha$  levels in *Grn*<sup>-/-</sup> mice, but had little effects in wild-type mice (Fig. 5A). To determine whether the anti-inflammatory effects of ABT-107 were associated with behavioral improvement, we tested these mice in a test of compulsivity, a symptom often associated with FTD [26]. We measured the amount of food that mice burrowed out of a canister placed in their home cage overnight, and found that *Grn*<sup>-/-</sup> mice burrowed significantly more than wild-type mice (Fig. 5B). However, treatment with ABT-107 significantly reduced the amount of food burrowed by *Grn*<sup>-/-</sup> mice compared with vehicle treatment, suggesting protection against FTD-related compulsivity



**Fig. 4.** Selective  $\alpha 7$  nAChR agonists decrease IL-1 $\beta$  and TNF $\alpha$ . (A–B) Nine-month-old progranulin knock-out mice were treated with vehicle, nicotine, or PHA-568487 by Alzet osmotic mini-pumps for 14 days. mRNA levels of IL-1 $\beta$  (A) or TNF $\alpha$  (B) were measured by qRT-PCR from brains and normalized to GAPDH mRNA levels.  $n = 9$ –10 mice per group. \* $P < 0.05$ , \*\*\* $P < 0.001$  by ANOVA followed by Tukey-Kramer *post-hoc* test. (C–D) Twelve-month-old progranulin knock-out mice were treated with vehicle, nicotine, or ABT-107 by Alzet osmotic mini-pumps for 14 days. mRNA levels of IL-1 $\beta$  (C) and TNF $\alpha$  (D) were measured by qRT-PCR from brains and normalized to GAPDH mRNA levels. Age-matched non-treated wild-type mice were used as control.  $n = 8$ –13 mice per group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by ANOVA followed by Tukey-Kramer *post-hoc* test.



**Fig. 5.** Selective  $\alpha 7$  nAChR agonist ABT-107 decreases TNF $\alpha$  and rescues compulsive behavior. (A–D) Sixteen-month-old wild-type or progranulin knock-out mice were injected daily (i.p.) with vehicle or ABT-107 for 14 days. (A) mRNA levels of TNF $\alpha$  were measured by qRT-PCR from brains and normalized to GAPDH mRNA levels.  $n = 5$ –7 mice per group.  $***P < 0.001$  by two-way ANOVA followed by Bonferroni *post-hoc* test. (B–C) Mice were tested for burrowing behavior. The amount of food burrowed after 15 h was measured. Progranulin knock-out mice burrowed more than wild-type mice (B).  $n = 5$ –6 mice per group.  $*P < 0.05$  by student's *t*-test. (C) Vehicle or ABT-107-treated mice were tested for burrowing behavior.  $n = 5$ –8 mice per group.  $*P < 0.05$  by two-way ANOVA followed by Bonferroni *post-hoc* test. (D) The amount of food burrowed was correlated with increased TNF $\alpha$  mRNA levels.  $n = 25$  mice,  $**P < 0.01$  with Gaussian approximation,  $r^2 = 0.2730$ .

(Fig. 5C). Notably, there was a significant positive correlation between the amount burrowed and TNF $\alpha$  levels in the brains of these mice, supporting a role for TNF $\alpha$  in progranulin deficiency-induced behavioral impairment (Fig. 5D).

#### 4. Discussion

Consistent with previous studies [27,28], we found that nicotine or  $\alpha 7$  nAChR agonist treatment decreased NF- $\kappa$ B activation in progranulin-deficient macrophages. Specific  $\alpha 7$  nAChR agonist PHA-568487 appeared to be more potent than nicotine in attenuating NF- $\kappa$ B activation induced by TNF $\alpha$  in cultured progranulin-deficient macrophages, supporting beneficial effects of selective activation of  $\alpha 7$  nAChRs. Interestingly, the two  $\alpha 7$  nAChR agonists we used exhibited differences as well. While both selective agonists significantly decreased microgliosis, PHA-568487 only decreased levels of IL-1 $\beta$ , but not TNF $\alpha$ , in progranulin-deficient mice (similar to nicotine), whereas ABT-107 appeared to decrease both. Although the three compounds could have differences in brain availability, some of the differential effects on cytokine release may also be reflective of the difference in the *in vitro* profiles of the compounds, with ABT-107 being 100-fold more potent to bind to  $\alpha 7$  nAChRs ( $K_i$  of 0.2–0.6 nM vs. 44 nM) and  $\sim 5$ -fold more potent to activate  $\alpha 7$  receptors with  $EC_{50}$  of 50 nM vs. 258 nM [29,30]. Both compounds also exhibit *in vivo* efficacy with PHA-568487 attenuating spatial memory deficits in a mouse model reflective of the cognitive impairment associated with schizophrenia [31], and ABT-107 exhibiting pre-clinical *in vivo* efficacy across a battery of assays associated with discrete cognitive domains [32]. In this latter study, ABT-107 was also shown to be neuroprotective via GSK $\beta$  inhibition and distinguished itself from nicotine as it did not induce behavioral sensitization or induce psychomotor stimulation in rats [32]. An additional  $\alpha 7$  agonist, A-833834, potently suppressed TNF $\alpha$  release via *in vitro* LPS induction or *in vivo* by zymosan-induced peritonitis [33]. The current data supports a role for  $\alpha 7$  nAChR activation in decreasing pro-inflammatory cytokines.

It has been previously demonstrated that modulation of TNF $\alpha$  release and subsequent downstream NF- $\kappa$ B activation is mediated by agonist activation of  $\alpha 7$  nAChRs [34]. Selective  $\alpha 7$  nAChR agonists can also inhibit pro-inflammatory cytokines by blocking phosphorylation of STAT3, which in turn prevents NF- $\kappa$ B activity [35]. In addition,  $\alpha 7$  agonists were shown to be neuroprotective in

an ischemic stroke injury model, where PHA-568487 reversed sensory motor deficits and attenuated lesion volume and neuronal apoptosis via reduction of CD68 $^+$  macrophages, activation of anti-oxidant genes, and reduced NF- $\kappa$ B activity in the affected infarct brain region [36]. In an Alzheimer's disease mouse model, microglial activation caused an upregulation of  $\alpha 7$  nAChRs, which could contribute to suppression of TNF $\alpha$  production [37]. This would support previous studies demonstrating that activation of  $\alpha 7$  nAChRs on microglia is neuroprotective in brain ischemia via induction of Nrf2 anti-oxidant genes [38]. Collectively, these reports combined with the current study using selective  $\alpha 7$  agonists continue to support the neuroprotective and anti-inflammatory properties of these compounds.

Here, we demonstrate a new phenotype in progranulin-deficient mice in the burrowing test, a measure of repetitive and compulsive activities and stereotyped behavior that has been used to characterize activities of daily living (ADLs) in mice [18,39,40]. Thus far, the primary behavior test that has been used to characterize FTD-associated behavior deficits in mice has been the three-chambered social test, which is a complex test that can be susceptible to numerous variables including lighting, time of day, age and sex of the stranger mouse, and experimenter error [5,23,41]. In contrast, mice display natural burrowing behavior that can be captured in a simple test that requires minimal experimenter handling. Of note, burrowing is commonly used to assess obsessive compulsive disorder (OCD)-like behaviors in rodents [42], and OCD-like symptoms are common and constitute a subset of criteria for diagnosis in behavioral variant FTD (bvFTD) [26,43]. Indeed, progranulin-deficient mice exhibited an increased burrowing phenotype, which was reversed by ABT-107. Although previous studies indicated decreased burrowing in mice in response to LPS administration, our data support that a chronic inflammatory state may actually lead to increases in compulsive behaviors [44,45].

The selective effect of ABT-107 on TNF $\alpha$  levels is intriguing—TNF $\alpha$  is an important inflammatory factor, but it has also been implicated in modulating neuronal and synaptic function [46–48]. TNF $\alpha$  is consistently and dramatically increased in progranulin-deficient mice [4,6,16,23], suggesting that it may play an integral role in mediating synaptic deficits underlying behavioral changes in these mice. Here, we provide evidence that ABT-107 markedly decreases TNF $\alpha$  levels, and this decrease is significantly correlated with improved burrowing behavior, demonstrating for the first



time a link between inflammation and FTD-like behavior deficits. However, we cannot discount the possibility that the anti-inflammatory effects of cholinergic agonists are distinct from the effects on neuronal function that drive behavioral changes. Since  $\alpha 7$  nAChRs are present on both neurons and microglia, activating the cholinergic system may benefit both pathways separately and, furthermore, this two-pronged approach may attenuate the reciprocal detrimental effects that each has on the other. Future studies will be necessary to establish the causality between microglial inflammation and neuronal dysfunction and behavioral outcome, especially in the context of progranulin-deficiency-associated FTD.

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