Perampanel Inhibits α-Synuclein Transmission in Parkinson's Disease Models

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ABSTRACT: Background: The intercellular transmission of pathogenic proteins plays a key role in the clinicopathological progression of neurodegenerative diseases. Previous studies have demonstrated that this uptake and release process is regulated by neuronal activity.
Objective: The objective of this study was to examine the effect of perampanel, an antiepileptic drug, on α-synuclein transmission in cultured cells and mouse models of Parkinson's disease.
Methods: Mouse primary hippocampal neurons were transduced with α-synuclein preformed fibrils to examine the effect of perampanel on the development of α-synuclein pathology and its mechanisms of action. An α-synuclein preformed fibril-injected mouse model was used to validate the effect of oral administration of perampanel on the α-synuclein pathology in vivo.
Results: Perampanel inhibited the development of α-synuclein pathology in mouse hippocampal neurons transduced with α-synuclein preformed fibrils. Interestingly, perampanel blocked the neuronal uptake of α-synuclein preformed fibrils by inhibiting macropinocytosis in a neuronal activity-dependent manner. We confirmed that oral administration of perampanel ameliorated the development of α-synuclein pathology in wild-type mice inoculated with α-synuclein preformed fibrils.
Conclusion: Modulation of neuronal activity could be a promising therapeutic target for Parkinson's disease, and perampanel could be a novel disease-modifying drug for Parkinson's disease.

Key Words: Parkinson's disease; α-synuclein; neuronal activity; perampanel; macropinocytosis

Parkinson's disease (PD) is pathologically characterized by progressive neuronal degeneration and the presence of Lewy bodies, which are composed of misfolded α-synuclein (α-syn). There is currently no therapy that inhibits or even slows the progression of PD. Based on postmortem analysis, Braak et al proposed a pathological staging of PD, in which the Lewy pathology in PD starts from the olfactory bulb (OB), anterior olfactory nucleus (AON), and dorsal nucleus of the vagus nerve and then spreads stereotypically to other interconnected brain regions.[1] Accumulating evidence suggests that misfolded α-syn behaves in a prion-like fashion and plays a significant role in PD progression.[2-14] Moreover, other pathogenic proteins, such as amyloid-β (Aβ) and tau in Alzheimer's disease (AD), are also thought to propagate in the brain and contribute to disease progression.[15] Although previous studies have revealed that exogenous α-syn preformed fibrils (PFFs) induce the propagation of α-syn pathology in cultured neurons[9] and mouse brains,[4,5,16] the molecular mechanisms

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and modulating factors underlying the propagation of α-syn pathology remain poorly understood.

Interestingly, a recent study demonstrated that the extracellular α-syn levels and α-syn release are affected by neuronal activity.[17] Moreover, extracellular release of tau, the formation of Aβ plaque, and the propagation of tau in AD are also affected by neuronal activity.[18-20] We hypothesized that the inhibition of neuronal activity could modulate the dynamics of α-syn, inhibit the propagation of α-syn pathology, and attenuate the progression of PD.

Perampanel (PER) is an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist that inhibits neuronal activity by blocking AMPA receptor-induced sodium and calcium influx into neurons.[21-23] PER has been shown to equipotently inhibit AMPA receptors in both glutamatergic and GABAergic neurons, and suppression of neuronal activity by PER has been demonstrated in previous in vitro and ex vivo studies.[21-26] In the present study, we examined whether the inhibition of neuronal activity by PER could attenuate the propagation of α-syn pathology.

Materials and Methods

Animals

C57BL/6J 3-month-old male mice (n = 46) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), or CLEA Japan, Inc (Osaka, Japan). All breeding, housing, and experimental procedures were conducted according to the guidelines for animal care of Kyoto University and were approved by the Kyoto University Animal Care and Use Committee.

Preparation of Recombinant α-Syn Monomers and PFFs

Mouse α-syn PFFs were generated as described previously.[27] We sonicated α-syn PFFs for 10 minutes (30-second sonication followed by an interval of 30 seconds was repeated 10 times, for a total of 10 minutes) with a Bioruptor bath sonicator before the administration of α-syn PFFs.

Stereotaxic Injection

Stereotaxic injection was performed as previously described.[28,29] The 3-month-old male mice anesthetized with Avertin (1.875% [w/v] 2,2,2-tribromoethanol, 1.25% [v/v] 3-methyl-1-butanol) were stereotaxically injected with 0.5 μL of α-syn PFFs (5 mg/mL) bilaterally into the OB (coordinates: AP, +4.5 mm; L or R, −0.9 mm; DV, −1.5 mm relative to the bregma and scull surface) using a 33-gauge microsyringe.

PER Treatment

PER powder (Eisai Co., Ltd.; Tokyo, Japan) was suspended in a 0.5% (w/v) methyl cellulose solution (final concentration of PER, 2.0 mg/mL; Wako; Osaka, Japan), and 10 μL/g of body weight was orally administered to the mice daily. The 3-month-old male mice were initially treated with 20 mg/kg PER before injection of α-syn PFFs (PER [pre], n = 8), 20 mg/kg PER after injection of α-syn PFFs (PER [post], n = 6), or vehicle before injection of α-syn PFFs (control, n = 7). The dose of PER was determined according to previous reports.[30,31] Treatment with PER or vehicle was continued for 2 weeks after injection of α-syn PFFs.

Immunohistochemistry

Immunohistochemistry was performed as previously described, with minor modifications.[27,28] Briefly, mice were sacrificed 2 weeks after injection of α-syn PFFs. The brains were fixed with 4% paraformaldehyde (PFA), embedded in paraffin, and processed to prepare 8-μm sections. An antibody against phosphorylated-α-syn (p-α-syn; 1:5000; ab51253, Abcam; Cambridge, England) was used as the primary antibody. The areas of p-α-syn-positive pathology in the AON and piriform cortex (PC) were quantified using the ImageJ software. For the assessment of AON, the total p-α-syn-positive areas and total numbers of neuronal p-α-syn-positive aggregates were evaluated in the images of 3 coronal sections at +3.08, +2.80, and +2.58 mm relative to the bregma. For the assessment of PC, the total p-α-syn-positive areas and total numbers of neuronal p-α-syn-positive aggregates were evaluated in the images of 4 coronal sections at +1.78, +0.38, −0.94, and −2.30 mm relative to the bregma.

Sequential Extraction

Sequential extraction of brain lysates was performed as previously described.[32] For biochemical analysis, we dissected the ventral half of the cerebral cortex containing the AON and PC from phosphate-buffered saline (PBS)–perfused brains of mice treated with 20 mg/kg PER or vehicle for 2 weeks without α-syn PFFs inoculation (all n = 5; Fig. 4A,B) or mice treated with 20 mg/kg PER or vehicle for 2 weeks after injection of α-syn PFFs into the OB (all n = 5; Fig. 4G).

Western Blotting

Western blotting was performed as previously described, with minor modification.[33] Briefly, 10 μg of Triton X–soluble or Triton X–insoluble samples was dissolved in sample buffer (1% [w/v] sodium dodecyl sulfate [SDS], 12.5% [w/v] glycerol, 0.005% [w/v] bromophenol blue, 2.5% [w/v] 2-mercaptoethanol, 25 mM Tris–HCl, pH 6.8) and separated on

10%–20% (w/v) gradient gels (FUJIFILM Wako Pure Chemical Corporation; Osaka, Japan). The proteins were transferred to polyvinylidene difluoride membranes (Merck Millipore; Burlington, MA). The membranes were treated with 4% (w/v) PFA in PBS for 30 minutes at room temperature (RT) before blocking to prevent detachment of α-syn from the blotted membranes. After blocking for 1 hour with 5% [w/v] skim milk in TBS-T, the membranes were incubated with primary antibodies against α-syn (1:2000; 610787; BD Biosciences; Franklin Lakes, NJ), β-actin (1:5000; A5441; Sigma-Aldrich; St. Louis, MO), and p-α-syn (1:5000; ab51253; Abcam; Cambridge, England) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (NB7574 or NB7160; Novus Biologicals; Centennial, CO) for 1 hour at RT. Immunoreactive bands were detected using detection reagent (Thermo Fisher Scientific; Waltham, MA), and the chemiluminescent signal was detected with Amersham Imager 600 (GE Healthcare; Chicago, IL). The band intensities were normalized to those of β-actin.

Primary Hippocampal Culture

Primary hippocampal cell cultures were prepared from E16 ICR mice. The embryos were removed and decapitated, and the entire hippocampus was dissected under sterile conditions. After enzymatic digestion for 5 minutes by 0.25% trypsin at 37°C, the cells were separated by trituration in Dulbecco's modified Eagle's medium (Wako; Osaka, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific; Waltham, MA) and 1% penicillin–streptomycin (Thermo Fisher Scientific; Waltham, MA). After trituration, the solution was centrifuged at 190*g* for 3 minutes, and the cell pellet was immediately resuspended in Neurobasal medium (Thermo Fisher Scientific; Waltham, MA) with 2% B27 (Invitrogen; Waltham, MA), 2 mM L-glutamine (Nacalai Tesque; Kyoto, Japan), and 1% penicillin–streptomycin (Thermo Fisher Scientific; Waltham, MA). The dissociated cells were plated on 24-well plates (1.5×10^5 cells/well) that were precoated with poly-DL-ornithine hydrobromide (Sigma-Aldrich; St. Louis, MO). Half the medium was removed and replaced every 3–4 days. The cells were cultured under constant conditions of 37°C, 5% CO_2 in a humidified incubator. The experiments were conducted over 14–17 days in vitro (DIV), and each experiment was repeated 3 times.

Cytotoxicity with Media LDH Assay

Cytotoxicity was assessed using media lactate dehydrogenase (LDH) assay kit (Cytotoxicity LDH Assay Kit-WST, Dojindo; Kumamoto, Japan). Supernatant (100 μL) was incubated with an equal volume of assay buffer for 30 minutes, and the absorbance of the culture medium was measured using a microplate reader at a test wavelength of 490 nm.

α-Syn PFFs, pHrodo-PFFs, and pHrodo-Dextran Transduction

Sonicated α-syn PFFs were labeled with pHrodo Red (Invitrogen; Waltham, MA), as per the manufacturer's instruction. The α-syn PFFs (final concentration, 0.05 μg/mL), α-syn PFFs labeled with pHrodo Red (pHrodo-PFFs; final concentration, 0.5 μg/mL), and pHrodo Red-dextran (10 kDa; Invitrogen; Waltham, MA; pHrodo-dextran; final concentration, 0.5 μg/mL) were added to the primary hippocampal culture at 14 DIV with PER (0.3, 3, 10, or 30 μM), 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX; 50 μM; Abcam; Cambridge, England), tetrodotoxin (TTX; 1 μM; Nacalai Tesque; Kyoto, Japan), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 50 μM; Cayman Chemical; Ann Arbor, MI), or vehicle and then incubated for the indicated time. The dose of PER was determined according to previous reports.[21,23] Primary neurons transduced with PBS alone were used as negative controls.

Immunocytochemistry

For immunocytochemistry, the cells were washed twice with PBS and then fixed with 4% (w/v) PFA in PBS for 5–20 minutes. After washing twice with PBS, incubation with PBS/0.1% Tween (10 minutes), and blocking with 3% (w/v) bovine serum albumin/PBS (1 hour at RT), the cells were incubated with primary antibodies against p-α-syn (1:3000; ab51253; Abcam; Cambridge, England), glial fibrillary acidic protein (GFAP; 1:500; M1406; Sigma-Aldrich; St Louis, MO), and neuronal nuclei (NeuN; 1:500; ABN78; Merck Millipore; Burlington, MA) at 4°C overnight. After washing with PBS, the cells were incubated with Alexa Fluor 488–conjugated (1:1000; A11001; Invitrogen; Waltham, MA), Alexa Fluor 594–conjugated (1:1000; A11037; Invitrogen; Waltham, MA), or Alexa Fluor 647–conjugated secondary antibodies (1:1000; A21094; Life Technologies; Carlsbad, CA) for 1 hour at RT. After washing with PBS and coverslipping, the cells were observed with BZ-X710 (Keyence; Osaka, Japan) at 20× magnification. The image acquisition settings were kept constant in all groups for each experiment. The number of NeuN-positive cells per field were counted to measure neuronal density. The areas of p-α-syn-positive pathology, pHrodo-PFFs, and pHrodo-dextran were quantified using ImageJ software. The average areas of p-α-syn-positive pathology, pHrodo-PFFs, and pHrodo-dextran per field (3–10 fields of view per sample) were averaged for the same conditions.

Statistical Analysis

Statistical analysis was conducted using PRISM statistical package. Statistical significance was evaluated by employing the Kruskal–Wallis test, followed by Dunn's post hoc test. The Mann–Whitney test was employed to compare the 2 groups of data. Statistical significance was set at $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

Results

PER Inhibits the Development of p-α-Syn-Positive Pathology in Hippocampal Primary Neurons

To investigate the potential effect of PER on PD pathology, we first assessed whether PER is effective against the development of p-α-syn-positive pathology using an in vitro PD model. In contrast to physiological α-syn, the majority of α-syn in Lewy pathology is phosphorylated at Ser129; thus, p-α-syn is a useful pathological marker of human PD and PD models.[4,5,10,34] Primary neurons transduced with α-syn PFFs exhibit p-α-syn-positive pathology and are well established as in vitro PD models to examine the mechanisms of the prion-like propagation of α-syn pathology.[9] In this study, mouse hippocampal primary neurons were transduced with α-syn PFFs in the presence of PER (0.3, 3, 10, or 30 μM) or vehicle at 14 DIV, followed by immunocytochemistry at 17 DIV. Primary neurons transduced with PBS alone were used as negative controls. To exclude the cytotoxic effect of PER, we measured neuronal density by counting the NeuN-positive cells, which revealed no significant difference among the groups (Fig. 1A). Next, we tested the effect of PER on the development of p-α-syn-positive pathology. Interestingly, immunocytochemistry revealed that less p-α-syn-positive pathology was observed in primary neurons transduced with α-syn PFFs in the presence of PER compared with those without PER (Fig. 1B,C). Primary neurons transduced with PBS alone exhibited no p-α-syn-positive pathology (Fig. 1C).

PER Inhibits the Activity-Dependent Uptake of α-Syn PFFs via Macropinocytosis in Hippocampal Primary Neurons

To elucidate the mechanisms of the decreased p-α-syn-positive pathology in α-syn PFFs-transduced primary neurons treated with PER, we investigated the potential effect of PER against the neuronal uptake of α-syn PFFs. We generated pHrodo-PFFs to examine the effect of PER on the uptake of α-syn PFFs in primary hippocampal neurons. Because of its favorable pH-sensitive photophysical properties, pHrodo Red is widely used for studying endocytosis.[35,36] In this study, at 14 DIV, primary hippocampal neurons were transduced with pHrodo-PFFs in the presence of PER (0.3, 3, 10, or 30 μM) or vehicle, incubated for 4 hours, followed by LDH assay, evaluation of the areas of pHrodo-PFFs and immunocytochemistry. Primary neurons transduced with PBS alone were used as negative controls. Neuronal density was measured to exclude cytotoxic effects of PER, and no significant difference was found among the groups (Fig. 2A). Moreover, LDH release into the conditioned medium did not differ among the groups (Fig. 2B), suggesting that PER treatment exhibited no appreciable toxicity to primary neurons within the 4-hour incubation period. Next, we assessed whether pHrodo-PFFs colocalize with NeuN, a neuronal marker, or GFAP, an astrocytic marker. Immunocytochemical analyses revealed only a small number of GFAP-positive cells, and those cells colocalized with pHrodo-PFFs compared with NeuN-positive cells (Fig. 2C, Fig. S1A,B). It has been reported that both astrocytes and neurons efficiently take up α-syn PFFs.[37] However, because the number of astrocytes was considerably lower than that of neurons in our primary neuronal culture (Fig. 2C, Fig. S1A,B), the fluorescence of pHrodo-PFFs was mostly observed in neurons. Therefore, the fluorescence of pHrodo-PFFs observed in this study can be considered as neuronal uptake of α-syn PFFs. We next tested the effect of PER on the neuronal uptake of α-syn PFFs. PER treatment decreased the pHrodo-PFFs areas compared with the control in a dose-dependent manner, indicating a reduction in the uptake of α-syn PFFs by these neurons (Fig. 2D,G), whereas primary neurons transduced with PBS alone exhibited no fluorescence (Fig. 2G). To confirm the mechanisms of action of PER, we tested the effect of NBQX, another AMPA receptor antagonist, and TTX, a sodium channel blocker, on the neuronal uptake of α-syn PFFs. Both NBQX (50 μM) and TTX (1 μM) treatment decreased the pHrodo-PFFs areas without toxicity (Fig. 2E,F, Figure S2A–D).

Although the mechanisms of α-syn PFFs uptake are not fully understood, several previous studies have demonstrated that α-syn PFFs uptake could be mediated by the endocytic process, including macropinocytosis.[36,38] Therefore, we investigated the effect of PER on macropinocytosis. First, we investigated whether macropinocytosis is involved in the neuronal α-syn PFFs uptake in the hippocampal primary neurons. EIPA is a specific inhibitor of macropinocytosis that blocks the Na^+/H^+ exchanger without affecting other endocytic pathways, such as clathrin-mediated endocytosis.[39-42] Hippocampal primary neurons transduced with pHrodo-PFFs in the presence of EIPA exhibited a remarkable decrease in pHrodo-PFFs areas without decreasing neuronal density (Fig. 3A–C). Next, we tested the efficacy of PER against macropinocytosis.

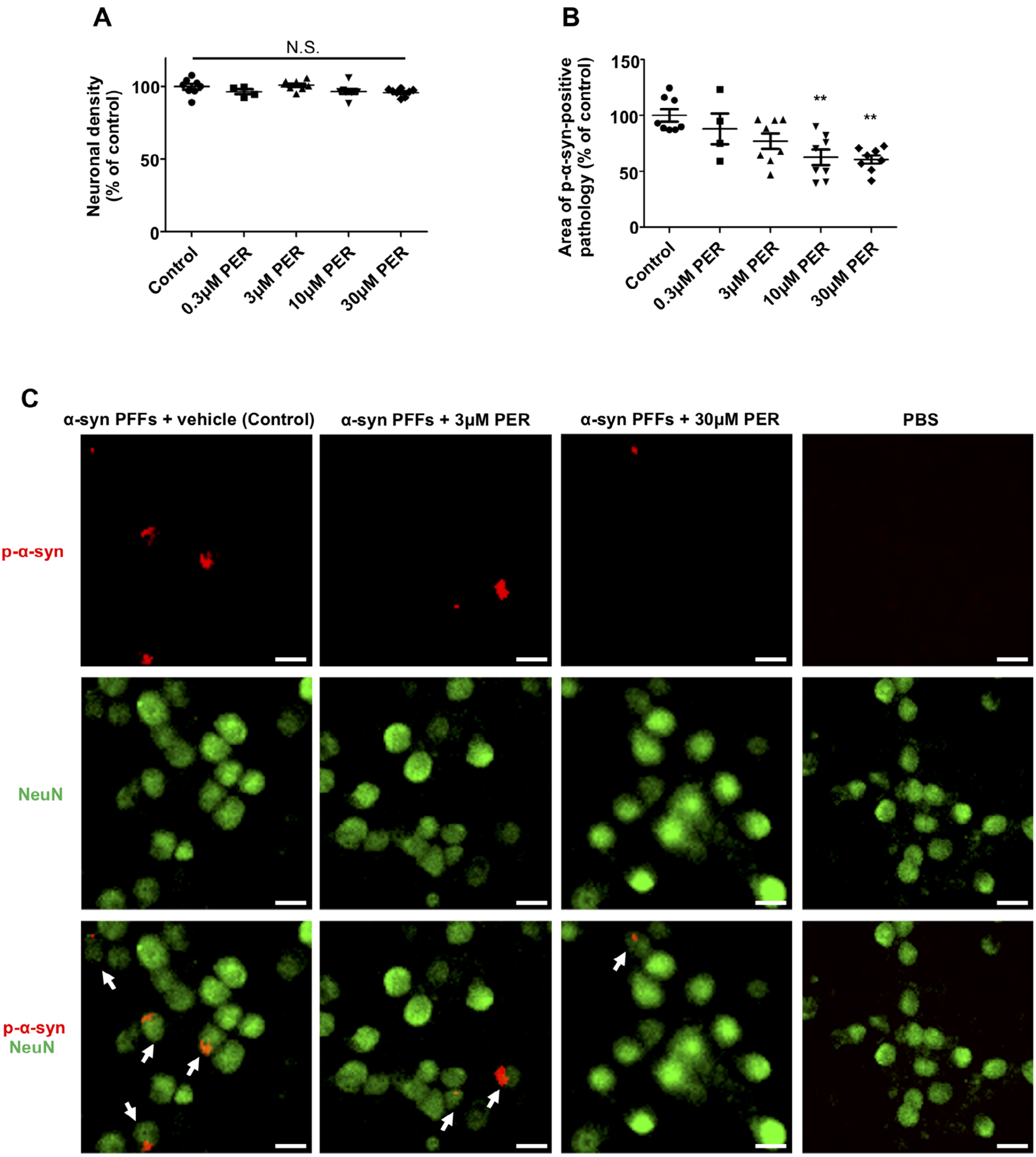

FIG. 1. PER inhibits the development of p-α-syn-positive pathology in primary hippocampal neurons. Control refers to the primary neurons that were transduced with α-syn PFFs and treated with vehicle. (A) Density of neurons. Data are representative of 3 independent experiments (n = 4–8). Data are normalized against the control and are expressed as mean ± SEM. N.S., not significant; Kruskal-Wallis test with Dunn's post hoc test. Scatterplots show data from each sample. (B) Area of p-α-syn-positive pathology in primary hippocampal neurons. Plotted data are representative of 3 independent experiments (n = 4–8). Data are normalized against the control and are expressed as mean ± SEM. $^{**}P < 0.01$, Kruskal–Wallis test with Dunn's post hoc test. (C) Representative images of immunohistochemical staining of primary hippocampal neurons. Data are representative of 3 independent experiments. Arrows indicate p-α-syn colocalization with NeuN-positive cells. Scale bar: 20 μm.

Dextran (10 kDa) is a marker of fluid-phase endocytosis; it is widely used to quantify macropinocytosis.[38-40] In this study, the hippocampal primary neurons were treated with pHrodo-dextran in the presence of PER (0.3, 3, 10, or 30 μM), NBQX (50 μM), TTX (1 μM), or vehicle at 14 DIV and then incubated for 4 hours. PER, NBQX, and TTX treatment resulted in decreased areas of pHrodo-dextran, indicating the inhibition of

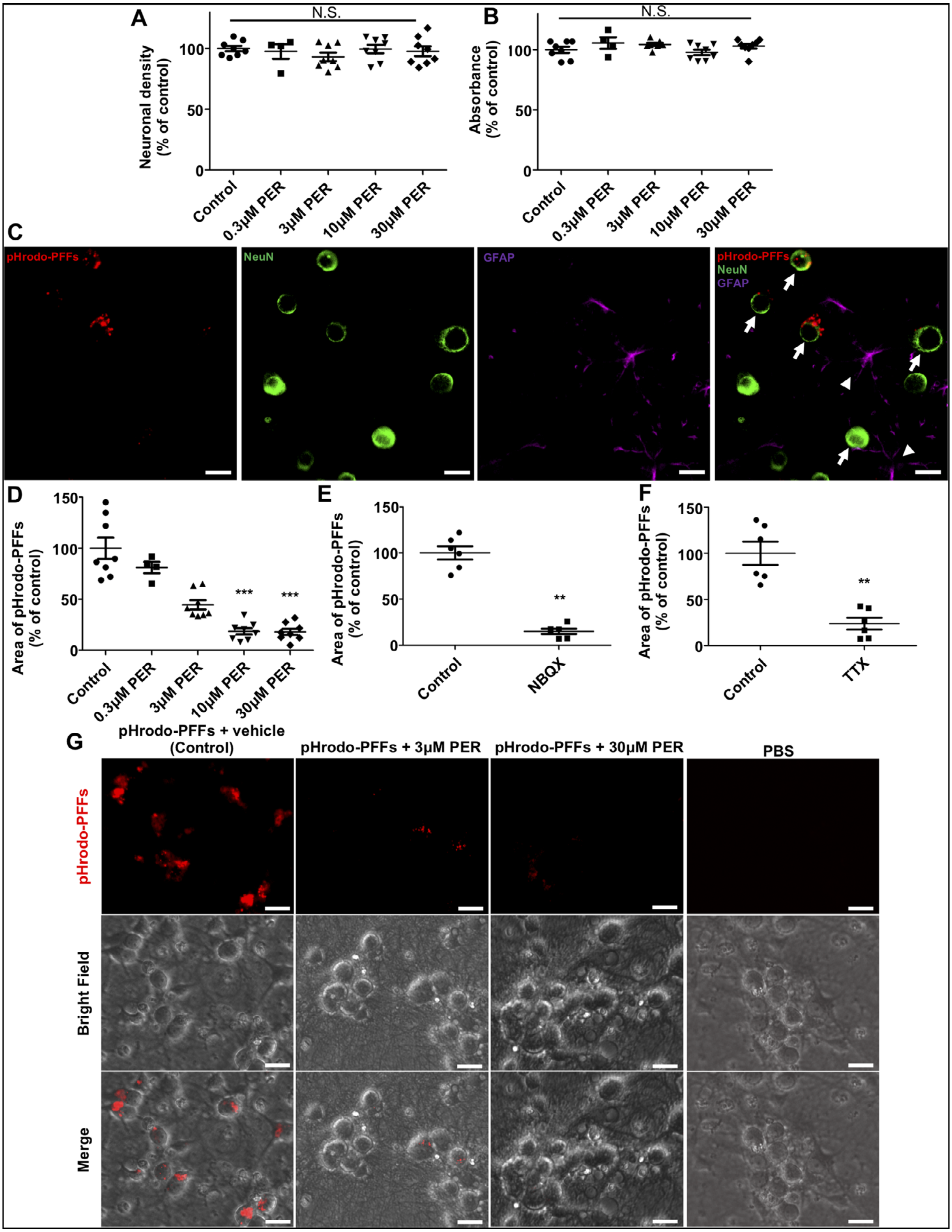

FIG. 2. PER, NBQX, and TTX inhibit the uptake of α-syn PFFs in primary hippocampal neurons. Control refers to the primary neurons that were transduced with pHrodo-PFFs and treated with vehicle. (A) Density of neurons. Data are representative of 3 independent experiments (n = 4–8). Data are normalized against the control and are expressed as mean ± SEM. N.S., not significant; Kruskal–Wallis test with Dunn's post hoc test. Scatterplots show data from each sample. (B) LDH assay. Plotted data are representative of 3 independent experiments (n = 4–8). Data are normalized against the control and are expressed as mean ± SEM. N.S., not significant; Kruskal–Wallis test with Dunn's post hoc test. (C) Representative images of immunohistochemical staining of primary hippocampal neurons. Data are representative of 3 independent experiments. Arrows indicate pHrodo-PFFs colocalized with NeuN-positive cells, and arrowheads indicate GFAP-positive cells. Scale bar: 20 μm. (D–F) Area of pHrodo-PFFs in primary hippocampal neurons. Plotted data are representative of 3 independent experiments (n = 4–8). Data are normalized against the control and are expressed as mean ± SEM. ***P* < 0.01, ****P* < 0.001; Kruskal–Wallis test with Dunn's post hoc test (D) and Mann–Whitney test (E, F). (G) Representative images of pHrodo-PFFs in primary hippocampal neurons. Data are representative of 3 independent experiments. Scale bar: 20 μm.

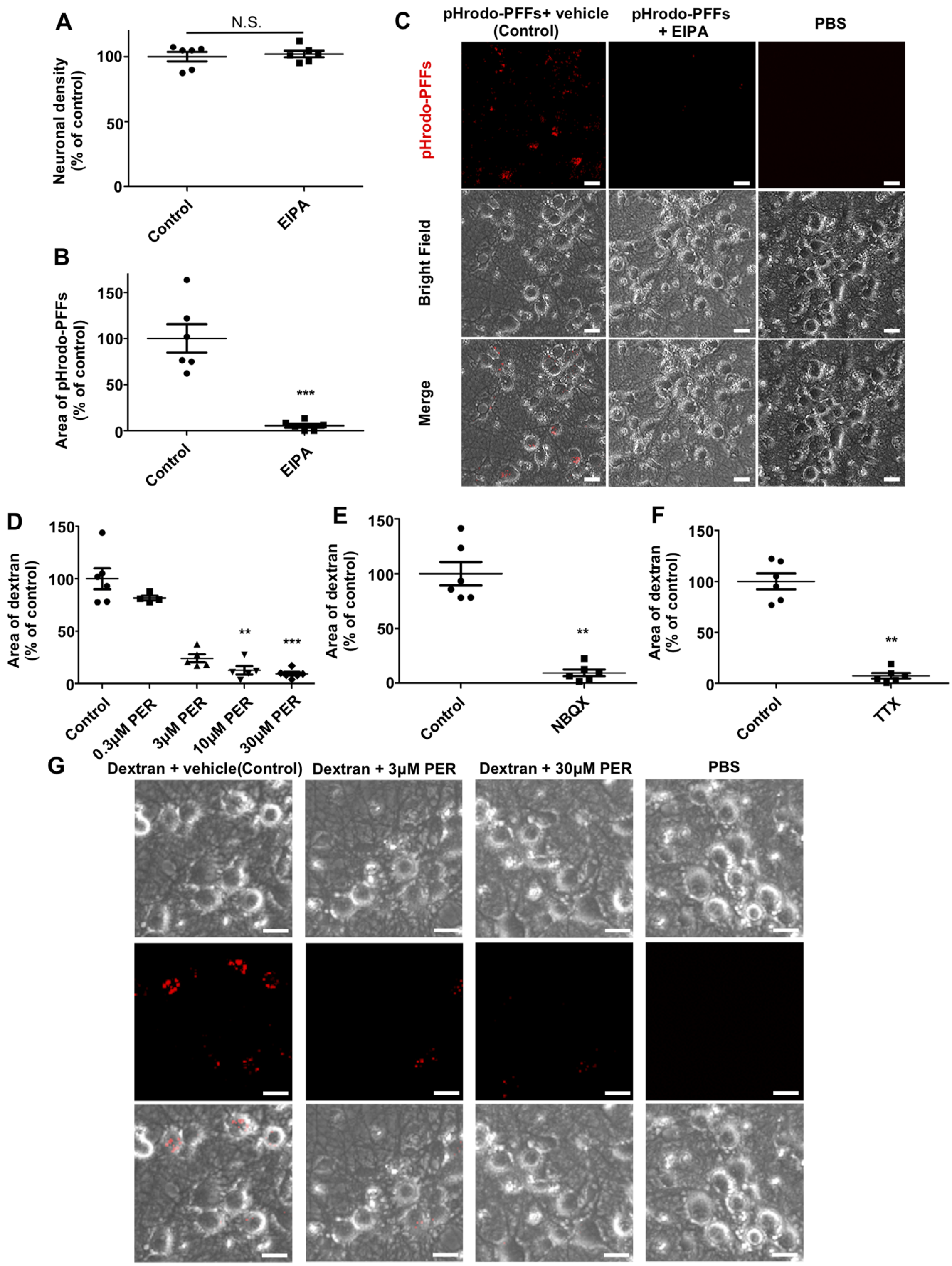

FIG. 3. PER, NBQX, and TTX inhibit the uptake of α-syn PFFs via macropinocytosis. (A) Density of neurons. Data are representative of 3 independent experiments (n = 6). Data are normalized against the control and are expressed as mean ± SEM. N.S., not significant; Mann–Whitney test. Scatterplots show data from each sample. (B) Area of pHrodo-PFFs in primary hippocampal neurons. Plotted data are representative of 3 independent experiments (n = 6). Data are normalized against the control and are expressed as mean ± SEM. $^{***}P < 0.001$; Mann–Whitney test. (C) Representative images of pHrodo-PFFs in primary hippocampal neurons. Data are representative of 3 independent experiments. Scale bar: 20 μm. (D–F) Area of pHrodo-dextran in primary hippocampal neurons. Plotted data are representative of 3 independent experiments (n = 4–6). Data are normalized against the control and are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$; Kruskal–Wallis test with Dunn's post hoc test (D), and Mann–Whitney test (E, F). (G) Representative images of pHrodo-dextran in primary hippocampal neurons. Data are representative of 3 independent experiments. Scale bar: 20 μm. A–C, control refers to the primary neurons that were transduced with pHrodo-PFFs and treated with vehicle; D–G, control refers to the primary neurons that were transduced with pHrodo-dextran and treated with vehicle.

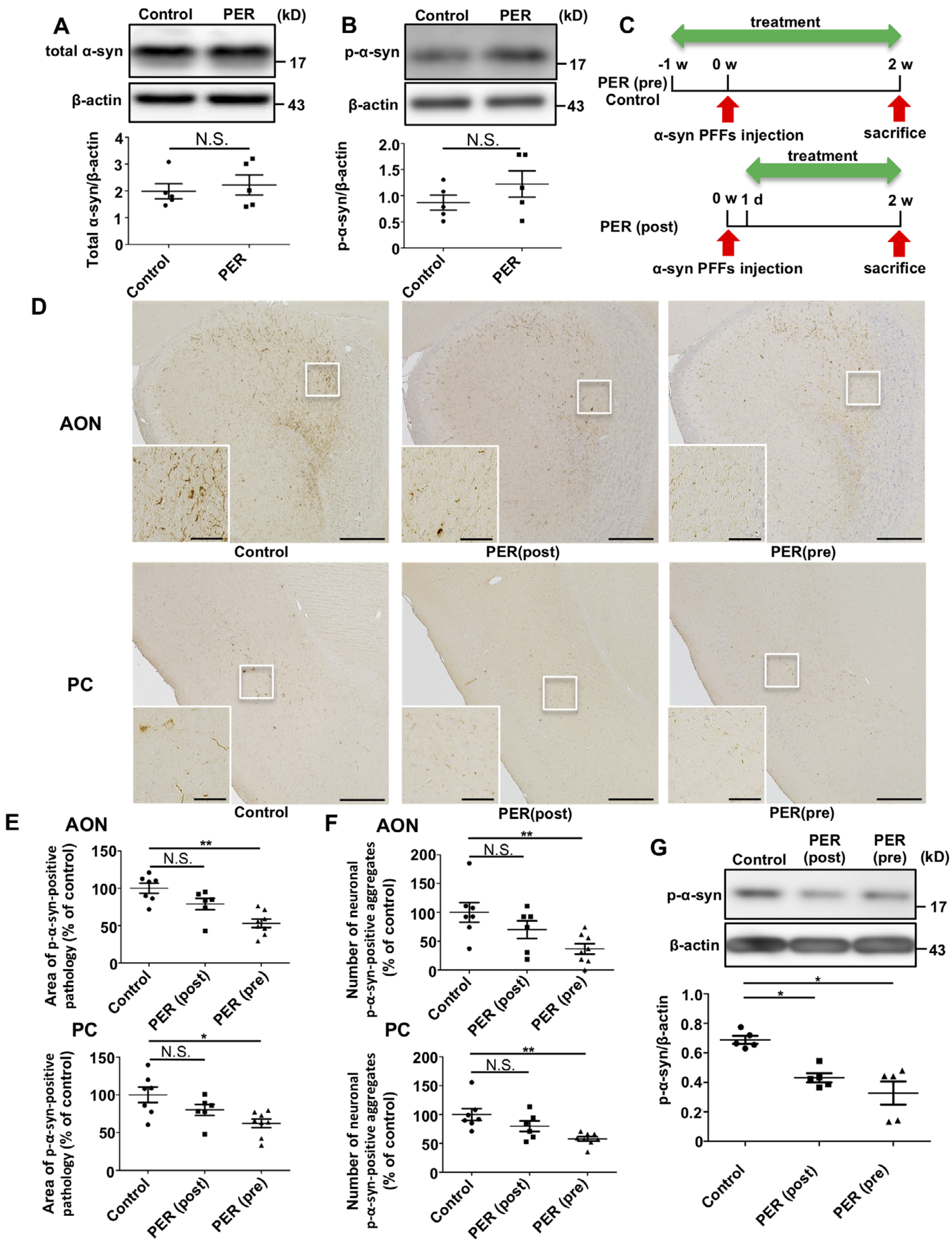

FIG. 4. PER inhibits the development of p-α-syn–positive pathology in a mouse model of PD. PER (pre) refers to "the mice in which PER treatment was initiated prior to the injection of α-syn PFFs," whereas PER (post) refers to "the mice in which PER treatment was initiated after the injection of α-syn PFFs." (A) Level of total α-syn in the Triton X–soluble fraction. The numbers (in kilodaltons) to the right indicate the position of the size markers. Representative images and plotted data are shown (n = 5). All values are expressed as mean ± SEM. N.S., not significant; Mann–Whitney test. (B) Level of p-α-syn in the Triton X–soluble fraction. The numbers (in kilodaltons) to the right indicate the position of the size markers. Representative images and plotted data are shown (n = 5). All values are expressed as mean ± SEM. N.S., not significant; Mann–Whitney test. (C) Time schedule for the injection of α-syn PFFs and drug treatment. (D) Representative images of immunohistochemical staining of the mice that underwent injection of α-syn PFFs and drug treatment. Insets show high-power images of p-α-syn-positive pathology in the AON and PC. Data are representative of 2 independent experiments. Scale bar: 200 μm (inset, 50 μm). (E) Area of p-α-syn-positive pathology in the AON and PC. Plotted data are pooled from 2 independent experiments (n = 6–8). Data are normalized against control and are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, N.S., not significant; Kruskal–Wallis test with Dunn's post hoc test. (F) Numbers of neuronal p-α-syn-positive aggregates in the AON and PC. Plotted data are pooled from 2 independent experiments (n = 6–8). Data are normalized against control and are expressed as mean ± SEM. **$P < 0.01$; N.S., not significant; Kruskal–Wallis test with Dunn's post hoc test. (G) Level of p-α-syn in the Triton X–insoluble fraction. The numbers (in kilodaltons) to the right indicate the position of the size markers. Representative images and plotted data are shown (n = 5). All values are expressed as mean ± SEM. *$P < 0.05$, Kruskal–Wallis test with Dunn's post hoc test.

macropinocytosis in hippocampal primary neurons (Fig. 3D–G).

PER Inhibits the Development of p-α-Syn-Positive Pathology in a Mouse PD Model

We further investigated the effect of PER on the propagation of α-syn pathology in a mouse PD model. First, we checked the expression of α-syn and p-α-syn in mouse brains by Western blot analysis to exclude the possibility that they are affected by PER administration. To this end, wild-type mice were treated orally with PER or vehicle for 2 weeks, and brain lysates containing AON and PC were sequentially extracted in Triton X and SDS buffers, followed by Western blotting. Western blot analysis revealed that PER had no significant effect on the expression of total α-syn and p-α-syn in the Triton X–soluble fraction (Fig. 4A,B).

Next, we examined whether PER treatment is also effective in an in vivo PD model. We previously reported that mice inoculated with α-syn PFFs into the OB, one of the initial lesions in PD, exhibited α-syn pathology mainly in the olfactory pathway, including the AON and PC, at 1 month postinoculation, but not in mice inoculated with PBS.[28] In this study, we analyzed wild-type mice inoculated with α-syn PFFs into the OB bilaterally by stereotaxic injections with or without oral administration of PER. PER treatment was initiated before or after the injection of α-syn PFFs, and mice were sacrificed 2 weeks after injection (Fig. 4C). In this study, PER (pre) refers to "the mice in which PER treatment was initiated before the injection of α-syn PFFs," whereas PER (post) refers to "the mice in which PER treatment was initiated after the injection of α-syn PFFs." Mice in which the treatment was started with vehicle alone before the injection of α-syn PFFs were used as a control group (Fig. 4C). We analyzed the areas of p-α-syn-positive pathology and the number of neuronal p-α-syn-positive aggregates in the AON and PC, as described previously.[28] In PER (pre), the areas of p-α-syn-positive pathology in the AON and PC were significantly decreased compared with those in the control (Fig. 4D,E); in PER (post), they were not significantly decreased compared with those in the control, although there was a tendency toward decreased p-α-syn-positive pathology (Fig. 4D,E). Moreover, the numbers of neuronal p-α-syn-positive aggregates in the AON and PC were also significantly decreased in PER (pre), but not in PER (post); see Figure 4F. We also investigated the amount of p-α-syn-positive aggregates by Western blot analysis. A previous study reported p-α-syn-positive bands in the detergent-insoluble fraction of mouse brains inoculated with α-syn PFFs by Western blot analysis.[5] In the current study, brain lysates containing the AON and PC of PER (pre), PER (post), or control were sequentially extracted in Triton X and SDS buffers, followed by Western blotting. In accordance with the immunohistochemical results, Western blot analysis showed significantly decreased p-α-syn in the Triton X–insoluble fraction of PER (pre) and PER (post) compared with that in the control (Fig. 4G).

Discussion

Although numerous studies have reported on the propagation of α-syn pathology in cultured neurons and mice, the correlation between the neuronal activity and the propagation of α-syn pathology remains unclear. Here we used in vitro and in vivo PD models to demonstrate that neuronal activity plays a crucial role in the propagation of α-syn pathology. We found that PER, as well as NBQX and TTX inhibit the neuronal uptake of α-syn PFFs and decrease the development of p-α-syn-positive pathology in primary neurons. PER and NBQX inhibit neuronal activity by blocking the AMPA receptor current,[23,43] whereas TTX suppresses neuronal activity in an AMPA receptor-independent manner by blocking the voltage-gated sodium channel. Thus, our results strongly suggest that the neuronal uptake of α-syn PFFs is mediated by an activity-dependent mechanism, and PER inhibits the formation of p-α-syn-positive pathology by reducing the activity-dependent neuronal uptake of α-syn PFFs. Another important finding is that the inhibitor of macropinocytosis remarkably decreased α-syn PFFs uptake, and PER, NBQX, and TTX inhibited macropinocytosis in primary neurons. Macropinocytosis is a type of fluid-phase endocytosis that is characterized by the formation of large endocytic vesicles termed macropinosomes (up to 5 μm). Previously, we demonstrated that the length of sonicated α-syn PFFs was 66.8 ± 3.1 nm (mean ± standard error of the mean [SEM]),[44] which suggests that a macropinosome is large enough for α-syn PFFs uptake. Although several studies have revealed that macropinocytosis could be involved in the uptake of pathogenic proteins in neurodegenerative diseases,[38-40] the correlation between macropinocytosis and neuronal activity has not yet been reported. Our results demonstrate that neuronal macropinocytosis is involved in the uptake of α-syn PFFs and is regulated, at least in part, by neuronal activity. Taken together, our in vitro results suggest that PER inhibits neuronal α-syn PFFs uptake by suppressing macropinocytosis in a neuronal activity-dependent manner.

Our in vivo results suggest that PER inhibits the development of p-α-syn pathology induced by α-syn PFFs without affecting the levels of total α-syn and p-α-syn expression, which is consistent with our in vitro results. Furthermore, our in vivo results also suggest that the presence or absence of PER treatment at the time of α-syn PFFs injection affects the development of p-α-syn-positive pathology (Fig. 4D–G). Because the neuronal uptake of α-syn PFFs is the initial step of propagation and starts immediately after α-syn PFFs injection,[45] the neuronal uptake of α-syn PFFs in PER (pre) could be more reduced than that in PER (post), leading to further reduction of p-α-syn-positive pathology in PER (pre). These results are consistent with the rapid transmission of α-syn PFFs via synaptic connections that was previously observed in a mouse PD model.[46] In this study, we assessed the neuronal uptake of α-syn PFFs and the initial development of α-syn pathology. However, because our in vivo PD model showed neuronal death more than 3 months after the injection of α-syn PFFs,[28] the duration of our in vivo study was insufficient to evaluate the long-term efficacy of PER. Further in vivo studies with longer follow-up are required to elucidate any negative effects of PER as well as to determine the long-term effect of PER on the subsequent propagation of α-syn pathology, neuronal death, and behavioral changes in PD models. Moreover, several clinical studies have reported that PER treatment has no beneficial effect on clinical symptoms in PD patients.[47,48] However, because the aim of these clinical studies was to evaluate the efficacy of PER against wearing off, the patients with PD were at an advanced stage and the duration of PER treatment was relatively short (≤30 weeks). To elucidate the disease-modifying effect of PER, de novo patients with PD should be treated with PER for a longer duration (eg, 36 months). After further validation of the effects of PER in animal studies, such clinical studies should be considered.

In conclusion, the major finding of this study is that PER inhibits the activity-dependent neuronal uptake of α-syn PFFs via macropinocytosis, and the subsequent development of p-α-syn-positive pathology in PD models. Our results support the idea that the propagation of α-syn pathology could be affected by an activity-dependent mechanism in neurons and suggest that PER could inhibit the neuronal transmission of pathogenic α-syn, thus slowing the progression of PD. Considering that neurodegenerative diseases have similar mechanisms of pathogenic protein transmission, PER could also be applied to other neurodegenerative diseases. Furthermore, because PER has already been approved as an antiepileptic drug in many countries, prompt clinical application for PD and other neurodegenerative diseases is possible. Targeting neuronal activity with PER could represent a new therapeutic strategy for synucleinopathies including PD and other neurodegenerative diseases.

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Author Contributions

J.U., M.S., N.U., H.Y. and R.T. designed the experiments. J.U. performed the experiments. J.U. and N.U. wrote the manuscript after a fruitful discussion with M. S., T.T., M.I., S.K., Y.T., S.M., H.Y.and R.T. All the authors have read and approved the final manuscript.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

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