

Perampanel inhibits α -synuclein transmission in Parkinson's disease models

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Word Count: 3,672 words

Running title: Perampanel inhibits α -synuclein transmission

21 **Keywords:** Parkinson's disease, alpha-synuclein, neuronal activity, perampanel,

22 macropinocytosis

23 **Relevant conflicts of interests/financial disclosures:** Nothing to report.

24 **Funding Sources:** This study was supported by a grant from the Ministry of Education,

25 Culture, Sports, Science, and Technology (No. 19K16610, RT, Grant-in-Aid for Scientific

26 Research (A); No. JP18H04041, Grant-in-Aid for Scientific Research on Innovative Areas;

27 No. JP17H05698), the Integrated Neurotechnologies for Disease Studies (Brain/MINDS)

28 from Japan Agency for Medical Research and Development AMED (RT, No.

29 JP18dm0207020 and JP19dm0207070).

30

31 **Abstract**

32 **Background:** The intercellular transmission of pathogenic proteins plays a key role in the

33 clinicopathological progression of neurodegenerative diseases. Previous studies have

34 demonstrated that this uptake and release process is regulated by neuronal activity.

35 **Objective:** To examine the effect of perampanel, an antiepileptic drug, on α -synuclein

36 transmission in cultured cells and mouse models of Parkinson's disease.

37 **Methods:** Mouse primary hippocampal neurons were transduced with α -synuclein
38 preformed fibrils to examine the effect of perampanel on the development of α -synuclein
39 pathology and its mechanisms of action. An α -synuclein preformed fibrils-injected mouse
40 model was used to validate the effect of oral administration of perampanel on the α -
41 synuclein pathology *in vivo*.

42 **Results:** Perampanel inhibited the development of α -synuclein pathology in mouse
43 hippocampal neurons transduced with α -synuclein preformed fibrils. Interestingly,
44 perampanel blocked the neuronal uptake of α -synuclein preformed fibrils by inhibiting
45 macropinocytosis in a neuronal activity-dependent manner. We confirmed that oral
46 administration of perampanel ameliorated the development of α -synuclein pathology in
47 wild-type mice inoculated with α -synuclein preformed fibrils.

48 **Conclusion:** Modulation of neuronal activity could be a promising therapeutic target for
49 Parkinson's disease, and perampanel could be a novel disease-modifying drug for
50 Parkinson's disease.

51

52 **Introduction**

53 Parkinson's disease (PD) is pathologically characterized by progressive neuronal
54 degeneration and the presence of Lewy bodies, which are composed of misfolded α -
55 synuclein (α -syn). There is currently no therapy that inhibits or even slows down the
56 progression of PD. Based on postmortem analysis, Braak et al. proposed a pathological
57 staging of PD, in which the Lewy pathology in PD starts from the olfactory bulb (OB),
58 anterior olfactory nucleus (AON), and dorsal nucleus of the vagus nerve and then spreads
59 stereotypically to other interconnected brain regions.¹ Accumulating evidence suggests
60 that misfolded α -syn behaves in a prion-like fashion and plays a significant role in PD
61 progression.²⁻¹⁴ Moreover, other pathogenic proteins, such as amyloid- β ($A\beta$) and tau in
62 Alzheimer's disease (AD), are also thought to propagate in the brain and contribute to
63 disease progression.¹⁵ Although previous studies have revealed that exogenous α -syn
64 preformed fibrils (PFFs) induce the propagation of α -syn pathology in cultured neurons⁹
65 and mouse brains,^{4, 5, 16} the molecular mechanisms and modulating factors underlying the
66 propagation of α -syn pathology remain poorly understood.

67 Interestingly, a recent study demonstrated that the extracellular α -syn levels and
68 α -syn release are affected by neuronal activity.¹⁷ Moreover, extracellular release of tau,
69 the formation of $A\beta$ plaque, and the propagation of tau in AD are also affected by neuronal

70 activity.^{18–20} We hypothesized that the inhibition of neuronal activity could modulate the
71 dynamics of α -syn, inhibit the propagation of α -syn pathology, and attenuate the
72 progression of PD.

73 Perampanel (PER) is an α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
74 (AMPA) receptor antagonist that inhibits neuronal activity by blocking the AMPA receptor-
75 induced sodium and calcium influx into neurons.^{21–23} PER has been shown to equipotently
76 inhibit AMPA receptors in both glutamatergic and GABAergic neurons, and suppression of
77 neuronal activity by PER has been demonstrated in previous *in vitro* and *ex vivo* studies.
78 ^{21–26} In the present study, we examined whether the inhibition of neuronal activity by PER
79 could attenuate the propagation of α -syn pathology.

80

81 **Materials and Methods**

82 **Animals**

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

87

88 **Preparation of recombinant α -syn monomers and PFFs**

89 Mouse α -syn PFFs were generated as described previously.²⁷ We sonicated α -syn PFFs
90 for 10 min (30-s sonication followed by an interval of 30 s, for a total of 10 min) with a
91 Bioruptor bath sonicator before the administration of α -syn PFFs.

92

93 **Stereotaxic injection**

94 Stereotaxic injection was performed as previously described.^{28, 29} The 3-month-old male
95 mice anesthetized with Avertin (1.875% [w/v] 2,2,2-tribromoethanol, 1.25% [v/v] 3-methyl-
96 1-butanol) were stereotaxically injected with 0.5 μ L of α -syn PFFs (5 mg/mL) bilaterally
97 into the OB (coordinates: AP: +4.5 mm, L or R: -0.9 mm, DV: -1.5 mm relative to the
98 bregma and skull surface) using a 33-gauge microsyringe.

99

100 **PER treatment**

101 PER powder (Eisai Co., Ltd.) was suspended in a 0.5% (w/v) methyl cellulose solution
102 (final concentration of PER: 2.0 mg/mL, Wako), and 10 μ L/g of body weight was orally
103 administered to the mice daily. The 3-month-old male mice were initially treated with 20

104 mg/kg PER before injection of α -syn PFFs (PER [pre], n = 8), 20 mg/kg PER after injection
105 of α -syn PFFs (PER [post], n = 6), or vehicle before injection of α -syn PFFs (control, n =
106 7). The dose of PER was determined according to previous reports.^{30, 31} Treatment with
107 PER or vehicle was continued for 2 weeks after injection of α -syn PFFs.

108

109 **Immunohistochemistry**

110 Immunohistochemistry was performed as previously described, with minor modifications.
111 ^{27, 28} Briefly, mice were sacrificed 2 weeks after injection of α -syn PFFs. The brains were
112 fixed with 4% paraformaldehyde (PFA), embedded in paraffin, and processed to prepare
113 8- μ m sections. An antibody against phosphorylated- α -syn (p- α -syn; 1:5000; ab51253,
114 Abcam) was used as the primary antibody. The areas of p- α -syn-positive pathology in the
115 AON and piriform cortex (PC) were quantified using the ImageJ software. For the
116 assessment of AON, the total p- α -syn-positive areas and total numbers of neuronal p- α -
117 syn-positive aggregates were evaluated in the images of three coronal sections at +3.08,
118 +2.80, and +2.58 mm relative to the bregma. For the assessment of PC, the total p- α -syn-
119 positive areas and total numbers of neuronal p- α -syn-positive aggregates were evaluated

120 in the images of four coronal sections at +1.78, +0.38, -0.94, and -2.30 mm relative to the
121 bregma.

122

123 **Sequential extraction**

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

130

131 **Western blotting**

132 Western blotting was performed as previously described, with minor modification.³³ Briefly,
133 10 μ g of Triton X-soluble or Triton X-insoluble samples was dissolved in sample buffer (1%
134 [w/v] sodium dodecyl sulfate [SDS], 12.5% [w/v] glycerol, 0.005% [w/v] bromophenol blue,
135 2.5% [w/v] 2-mercaptoethanol, 25 mM Tris-HCl, pH 6.8) and separated on 10%–20% (w/v)
136 gradient gels (FUJIFILM Wako Pure Chemical Corporation). The proteins were transferred
137 to polyvinylidene difluoride membranes (Merck Millipore). The membranes were treated
138 with 4% (w/v) PFA in PBS for 30 min at room temperature (RT) before blocking to prevent
139 detachment of α -syn from the blotted membranes. After blocking for 1 h with 5% [w/v] skim

140 milk in TBS-T, the membranes were incubated with primary antibodies against α -syn
141 (1:2000; 610787, BD Biosciences), β -actin (1:5000; A5441, Sigma-Aldrich), and p- α -syn
142 (1:5000; ab51253, Abcam) overnight at 4°C. Subsequently, the membranes were
143 incubated with horseradish peroxidase-conjugated secondary antibodies (NB7574 or
144 NB7160; Novus Biologicals) for 1 h at RT. Immunoreactive bands were detected using
145 detection reagent (Thermo Fisher Scientific), and the chemiluminescent signal was
146 detected with Amersham Imager 600 (GE Healthcare). The band intensities were
147 normalized to those of β -actin.

148

149 **Primary hippocampal culture**

150 Primary hippocampal cell cultures were prepared from E16 ICR mice. The embryos were
151 removed and decapitated, and the entire hippocampus was dissected under sterile
152 conditions. After enzymatic digestion for 5 min by 0.25% trypsin at 37°C, the cells were
153 separated by trituration in Dulbecco's modified Eagle's medium (Wako) supplemented with
154 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin–streptomycin
155 (Thermo Fisher Scientific). After trituration, the solution was centrifuged at 190 g for 3 min,
156 and the cell pellet was immediately re-suspended in Neurobasal medium (Thermo Fisher

Scientific) with 2% B27 (Invitrogen), 2 mM L-glutamine (Nacalai Tesque), and 1% penicillin–streptomycin (Thermo Fisher Scientific). The dissociated cells were plated on 24-well plates (1.5×10^5 cells/well) that were pre-coated with poly-DL-ornithine hydrobromide (Sigma-Aldrich). Half of the medium was removed and replaced every 3–4 days. The cells were cultured under constant conditions of 37°C, 5% CO₂ in a humidified incubator. The experiments were conducted over 14–17 days *in vitro* (DIV), and each experiment was repeated three times.

164

165 **Cytotoxicity with media LDH assay**

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

170

171 **α-syn PFFs, pHrodo-PFFs, and pHrodo-dextran transduction**

Sonicated α-syn PFFs were labeled with pHrodo Red (Invitrogen), as per the manufacturer's instruction. α-syn PFFs (final concentration: 0.05 µg/mL), α-syn PFFs

174 labeled with pHrodo Red (pHrodo-PFFs; final concentration: 0.5 µg/mL), and pHrodo Red-
175 dextran (10 kDa; Invitrogen), (pHrodo-dextran; final concentration: 0.5 µg/mL) were added
176 to the primary hippocampal culture at 14 DIV with PER (0.3, 3, 10, or 30 µM), 2,3-Dioxo-6-
177 nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; 50 µM, Abcam),
178 tetrodotoxin (TTX; 1 µM, Nacalai Tesque), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 50 µM,
179 Cayman Chemical), or vehicle and then incubated for the indicated time. The dose of PER
180 was determined according to previous reports.^{21, 23} Primary neurons transduced with PBS
181 alone were used as negative controls.

182

183 **Immunocytochemistry**

184 For immunocytochemistry, the cells were washed twice with PBS and then fixed with 4%
185 (w/v) PFA in PBS for 5–20 min. After washing twice with PBS, incubation with
186 PBS/0.1% Tween (10 min), and blocking with 3% (w/v) bovine serum albumin/PBS (1 h at
187 RT), the cells were incubated with primary antibodies against p-α-syn (1:3000; ab51253,
188 Abcam), glial fibrillary acidic protein (GFAP; 1:500; M1406, Sigma-Aldrich), and neuronal
189 nuclei (NeuN; 1:500; ABN78, Merck Millipore) at 4°C overnight. After washing with PBS,
190 the cells were incubated with Alexa Fluor 488-conjugated (1:1000; A11001, Invitrogen),

191 594-conjugated (1:1000; A11037, Invitrogen), or 647-conjugated secondary antibodies
192 (1:1000; A21094, Life Technologies) for 1 h at RT. After washing with PBS and cover-
193 slipping, the cells were observed with BZ-X710 (Keyence) at $\times 20$ magnification. The
194 image acquisition settings were kept constant in all groups for each experiment. The
195 number of NeuN-positive cells per field were counted to measure neuronal density. The
196 areas of p- α -syn-positive pathology, pHrodo-PFFs, and pHrodo-dextran were quantified
197 using ImageJ software. The average areas of p- α -syn-positive pathology, pHrodo-PFFs,
198 and pHrodo-dextran per field (3–10 fields of view per sample) were averaged for the same
199 conditions.

200

201 **Statistical analysis**

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

206

207 **Results**

208

209 **PER inhibits the development of p- α -syn-positive pathology in hippocampal primary**
210 **neurons**

211 To investigate the potential effect of PER on PD pathology, we first assessed whether
212 PER is effective against the development of p- α -syn-positive pathology using an *in vitro*
213 PD model. In contrast to physiological α -syn, the majority of α -syn in Lewy pathology is
214 phosphorylated at Ser129; thus, p- α -syn is a useful pathological marker of human PD and
215 PD models.^{4, 5, 10, 34} Primary neurons transduced with α -syn PFFs exhibit p- α -syn-positive
216 pathology and are well established as *in vitro* PD models to examine the mechanisms of
217 the prion-like propagation of α -syn pathology.⁹ In this study, mouse hippocampal primary
218 neurons were transduced with α -syn PFFs in the presence of PER (0.3, 3, 10, or 30 μ M) or
219 vehicle at 14 DIV, followed by immunocytochemistry at 17 DIV. Primary neurons
220 transduced with PBS alone were used as negative controls. To exclude the cytotoxic effect
221 of PER, we measured the neuronal density by counting the NeuN-positive cells, which
222 revealed no significant difference among the groups (Fig. 1A). Next, we tested the effect of
223 PER on the development of p- α -syn-positive pathology. Interestingly,
224 immunocytochemistry revealed that less p- α -syn-positive pathology was observed in

225 primary neurons transduced with α -syn PFFs in the presence of PER compared with those
226 without PER (Fig. 1B, C). Primary neurons transduced with PBS alone exhibited no p- α -
227 syn-positive pathology (Fig. 1C).

228

229 **PER inhibits the activity-dependent uptake of α -syn PFFs via macropinocytosis in**
230 **hippocampal primary neurons**

231 To elucidate the mechanisms of the decreased p- α -syn-positive pathology in α -syn PFFs-
232 transduced primary neurons treated with PER, we investigated the potential effect of PER
233 against the neuronal uptake of α -syn PFFs. We generated pHrodo-PFFs to examine the
234 effect of PER on the uptake of α -syn PFFs in primary hippocampal neurons. Due to its
235 favorable pH-sensitive photophysical properties, pHrodo Red is widely used for studying
236 endocytosis.^{35, 36} In this study, at 14 DIV, primary hippocampal neurons were transduced
237 with pHrodo-PFFs in the presence of PER (0.3, 3, 10, or 30 μ M) or vehicle, incubated for 4
238 h, followed by LDH assay, evaluation of the areas of pHrodo-PFFs, and
239 immunocytochemistry. Primary neurons transduced with PBS alone were used as negative
240 controls. The neuronal density was measured to exclude cytotoxic effects of PER, and no
241 significant difference was found among the groups (Fig. 2A). Moreover, LDH release into

242 the conditioned medium did not differ among the groups (Fig. 2B), suggesting that PER
243 treatment exhibited no appreciable toxicity to primary neurons within the 4-h incubation
244 period. Next, we assessed whether pHrodo-PFFs colocalize with NeuN, a neuronal
245 marker, or GFAP, an astrocytic marker. Immunocytochemical analyses revealed only a
246 small number of GFAP-positive cells and those cells colocalized with pHrodo-PFFs
247 compared to NeuN-positive cells (Fig. 2C, Supporting Information Fig. S1A, B). It has been
248 reported that both astrocytes and neurons efficiently take up α -syn PFFs.³⁷ However,
249 because the number of astrocytes was considerably lower than that of neurons in our
250 primary neuronal culture (Fig. 2C, Supporting Information Fig. S1A, B), the fluorescence of
251 pHrodo-PFFs was mostly observed in neurons. Therefore, the fluorescence of pHrodo-
252 PFFs observed in this study can be considered as neuronal uptake of α -syn PFFs. We
253 next tested the effect of PER on the neuronal uptake of α -syn PFFs. PER treatment
254 decreased the pHrodo-PFFs areas compared to the control in a dose-dependent manner,
255 indicating a reduction in the uptake of α -syn PFFs by these neurons (Fig. 2D, G), while
256 primary neurons transduced with PBS alone exhibited no fluorescence (Fig. 2G). To
257 confirm the mechanisms of action of PER, we tested the effect of NBQX, another AMPA
258 receptor antagonist, and TTX, a sodium channel blocker, on the neuronal uptake of α -syn

259 PFFs. Both NBQX (50 μ M) and TTX (1 μ M) treatment decreased the pHrodo-PFFs areas
260 without toxicity (Fig. 2E, F, Supporting Information Fig. S2A–D).

261 Although the mechanisms of α -syn PFFs uptake are not fully understood, several
262 previous studies have demonstrated that α -syn PFFs uptake could be mediated by the
263 endocytic process, including macropinocytosis.^{36, 38} Therefore, we investigated the effect
264 of PER on macropinocytosis. First, we investigated whether macropinocytosis is involved
265 in the neuronal α -syn PFFs uptake in the hippocampal primary neurons. EIPA is a specific
266 inhibitor of macropinocytosis that blocks the Na^+/H^+ exchanger without affecting other
267 endocytic pathways, such as clathrin-mediated endocytosis.^{39–42} Hippocampal primary
268 neurons transduced with pHrodo-PFFs in the presence of EIPA exhibited a remarkable
269 decrease in pHrodo-PFFs areas without decreasing neuronal density (Fig. 3A–C). Next,
270 we tested the efficacy of PER against macropinocytosis. Dextran (10 kDa) is a marker of
271 fluid phase endocytosis; it is widely used to quantify macropinocytosis.^{38–40} In this study,
272 the hippocampal primary neurons were treated with pHrodo-dextran in the presence of
273 PER (0.3, 3, 10, or 30 μ M), NBQX (50 μ M), TTX (1 μ M), or vehicle at 14 DIV and then
274 incubated for 4 h. PER, NBQX, and TTX treatment resulted in decreased areas of pHrodo-

275 dextran, indicating the inhibition of macropinocytosis in hippocampal primary neurons (Fig.
276 3D–G).

277

278 **PER inhibits the development of p- α -syn-positive pathology in a mouse PD model**

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

287 Next, we examined whether PER treatment is also effective in an *in vivo* PD
288 model. We previously reported that mice inoculated with α -syn PFFs into the OB, one of
289 the initial lesions in PD, exhibited α -syn pathology mainly in the olfactory pathway,
290 including the AON and PC, at 1 month post-inoculation, but not in mice inoculated with
291 PBS.²⁸ In this study, we analyzed wild type mice inoculated with α -syn PFFs into the OB

292 bilaterally by stereotaxic injections with or without oral administration of PER. PER
293 treatment was initiated before or after the injection of α -syn PFFs, and mice were
294 sacrificed 2 weeks after injection (Fig. 4C). In this study, “PER (pre)” refers to “the mice in
295 which PER treatment was initiated before the injection of α -syn PFFs,” whereas “PER
296 (post)” refers to “the mice in which PER treatment was initiated after the injection of α -
297 PFFs.” Mice in which the treatment was started with vehicle alone before the injection of α -
298 syn PFFs were used as a control group (Fig. 4C). We analyzed the areas of p- α -syn-
299 positive pathology and the number of neuronal p- α -syn-positive aggregates in the AON
300 and PC, as described previously.²⁸ In PER (pre), the areas of p- α -syn-positive pathology in
301 the AON and PC were significantly decreased compared with those in the control (Fig. 4D,
302 E); in PER (post), they were not significantly decreased compared with those in the
303 control, although there was a tendency toward decreased p- α -syn-positive pathology (Fig.
304 4D, E). Moreover, the numbers of neuronal p- α -syn-positive aggregates in the AON and
305 PC were also significantly decreased in PER (pre), but not in PER (post) (Fig. 4F). We
306 also investigated the amount of p- α -syn-positive aggregates by Western blot analysis. A
307 previous study reported p- α -syn-positive bands in the detergent-insoluble fraction of
308 mouse brains inoculated with α -syn PFFs by Western blot analysis.⁵ In the current study,

309 brain lysates containing the AON and PC of PER (pre), PER (post), or control were
310 sequentially extracted in Triton X and SDS buffers, followed by Western blotting. In
311 accordance with the immunohistochemical results, Western blot analysis showed
312 significantly decreased p- α -syn in the Triton X-insoluble fraction of PER (pre) and PER
313 (post) compared to that in the control (Fig. 4G).

314

315 **Discussion**

316 Although numerous studies have reported on the propagation of α -syn pathology in
317 cultured neurons and mice, the correlation between the neuronal activity and the
318 propagation of α -syn pathology remains unclear. Here we used *in vitro* and *in vivo* PD
319 models to demonstrate that neuronal activity plays a crucial role in the propagation of α -
320 syn pathology. We found that PER, as well as NBQX and TTX inhibit the neuronal uptake
321 of α -syn PFFs and decrease the development of p- α -syn-positive pathology in primary
322 neurons. PER and NBQX inhibit neuronal activity by blocking the AMPA receptor
323 current,^{23, 43} whereas TTX suppresses neuronal activity in an AMPA receptor-independent
324 manner by blocking the voltage-gated sodium channel. Thus, our results strongly suggest
325 that the neuronal uptake of α -syn PFFs is mediated by an activity-dependent mechanism,

326 and PER inhibits the formation of p- α -syn-positive pathology by reducing the activity-
327 dependent neuronal uptake of α -syn PFFs. Another important finding is that the inhibitor of
328 macropinocytosis remarkably decreased α -syn PFFs uptake, and PER, NBQX, and TTX
329 inhibited macropinocytosis in primary neurons. Macropinocytosis is a type of fluid phase
330 endocytosis that is characterized by the formation of large endocytic vesicles termed
331 macropinosomes (up to 5 μ m). Previously, we demonstrated that the length of sonicated
332 α -syn PFFs was 66.8 ± 3.1 nm (mean $\pm$ standard error of the mean [SEM]),⁴⁴ which
333 suggests that a macropinosome is large enough for α -syn PFFs uptake. Although several
334 studies have revealed that macropinocytosis could be involved in the uptake of pathogenic
335 proteins in neurodegenerative diseases,^{38–40} the correlation between macropinocytosis
336 and neuronal activity has not yet been reported. Our results demonstrate that neuronal
337 macropinocytosis is involved in the uptake of α -syn PFFs and is regulated, at least in part,
338 by neuronal activity. Taken together, our *in vitro* results suggest that PER inhibits neuronal
339 α -syn PFFs uptake by suppressing macropinocytosis in a neuronal activity-dependent
340 manner.

341 Our *in vivo* results suggest that PER inhibits the development of p- α -syn
342 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). Since 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, since 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, since the aim of these clinical studies was to evaluate the efficacy

360 of PER against wearing off, the patients with PD were at an advanced stage and the
361 duration of PER treatment was relatively short (≤ 30 weeks). In order to elucidate the
362 disease-modifying effect of PER, *de novo* patients with PD should be treated with PER for
363 a longer duration (e.g., 36 months). After further validation of the effects of PER in animal
364 studies, such clinical studies should be considered.

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

377

378 **Acknowledgments**

379 We wish to thank Yusuke Hatanaka Ph.D., Ms. Rie Hikawa and Ms. Ikuko Amano for the
380 technical assistance, and Eisai Co., Ltd. for providing us with PER.

381

382 **Author contributions**

383 JU, MS, NU, and HY designed the experiments. JU performed the experiments. JU and
384 NU wrote the manuscript after a fruitful discussion with MS, TT, MI, SK, YT, SM, and HY.
385 All the authors have read and approved the final manuscript. RT supervised all the
386 experiments.

387

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512

513 **FIGURE LEGENDS**

514

515 **FIG. 1**

516 PER inhibits the development of p- α -syn-positive pathology in primary hippocampal
517 neurons. (A) Density of neurons. In Fig. 1, “control” refers to the primary neurons that were
518 transduced with α -syn PFFs and treated with vehicle. Data are representative of three
519 independent experiments (n = 4–8). Data are normalized against control and are

520 expressed as mean $\pm$ SEM. N.S.: Not significant, Kruskal-Wallis test with Dunn's *post hoc*
521 test. Scatter plots show data from each sample. (B) Area of p- α -syn-positive pathology in
522 primary hippocampal neurons. Plotted data are representative of three independent
523 experiments (n = 4–8). Data are normalized against control and are expressed as mean $\pm$
524 SEM. $**P < 0.01$; Kruskal-Wallis test with Dunn's *post hoc* test. (C) Representative images
525 of immunohistochemical staining of primary hippocampal neurons. Data are representative
526 of three independent experiments. Arrows indicate p- α -syn colocalization with NeuN-
527 positive cells. Scale bar: 20 μ m.

528

529 **FIG. 2**

530 PER, NBQX, and TTX inhibit the uptake of α -syn PFFs in primary hippocampal neurons.

531 (A) Density of neurons. In Fig. 2, “control” refers to the primary neurons that were

532 transduced with pHrodo-PFFs and treated with vehicle. Data are representative of three

533 independent experiments (n = 4–8). Data are normalized against control and are

534 expressed as mean $\pm$ SEM. N.S.: Not significant, Kruskal-Wallis test with Dunn's *post hoc*

535 test. Scatter plots show data from each sample. (B) LDH assay. Plotted data are

536 representative of three independent experiments (n = 4–8). Data are normalized against

control and are expressed as mean $\pm$ 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 three independent experiments. Arrows indicate pHrodo-PFFs colocalized with NeuN-positive cells, and arrowheads indicate GFAP-positive cells. Scale bar: 20 μ m. (D) (E) (F) Area of pHrodo-PFFs in primary hippocampal neurons. Plotted data are representative of three independent experiments (n = 4–8). Data are normalized against control and are expressed as mean $\pm$ 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 three independent experiments. Scale bar: 20 μ m.

FIG. 3

PER, NBQX, and TTX inhibit the uptake of α -syn PFFs via macropinocytosis. (A) Density of neurons. In Fig. 3A–3C, “control” refers to the primary neurons that were transduced with pHrodo-PFFs and treated with vehicle. Data are representative of three independent experiments (n = 6). Data are normalized against control and are expressed as mean $\pm$ SEM. N.S.: Not significant, Mann Whitney test. Scatter plots show data from each sample.

554 (B) Area of pHrodo-PFFs in primary hippocampal neurons. Plotted data are representative
555 of three independent experiments ($n = 6$). Data are normalized against control and are
556 expressed as mean $\pm$ SEM. *** $P < 0.001$, Mann Whitney test. (C) Representative images
557 of pHrodo-PFFs in primary hippocampal neurons. Data are representative of three
558 independent experiments. Scale bar: 20 μm . (D) (E) (F) Area of pHrodo-dextran in primary
559 hippocampal neurons. In Fig. 3D–3G, “control” refers to the primary neurons that were
560 transduced with pHrodo-dextran and treated with vehicle. Plotted data are representative
561 of three independent experiments ($n = 4–6$). Data are normalized against control and are
562 expressed as mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$, Kruskal-Wallis test with Dunn’s *post*
563 *hoc* test (D), and Mann Whitney test (E, F). (G) Representative images of pHrodo-dextran
564 in primary hippocampal neurons. Data are representative of three independent
565 experiments. Scale bar: 20 μm .

566

567 **FIG. 4**

568 PER inhibits the development of p- α -syn–positive pathology in a mouse model of PD. (A)
569 Level of total α -syn in the Triton X-soluble fraction. The numbers (in kDa) to the right
570 indicate the position of the size markers. Representative images and plotted data are

571 shown ($n = 5$). All values are expressed as mean $\pm$ SEM. N.S.: Not significant, Mann
572 Whitney test. (B) Level of p- α -syn in the Triton X-soluble fraction. The numbers (in kDa) to
573 the right indicate the position of the size markers. Representative images and plotted data
574 are shown ($n = 5$). All values are expressed as mean $\pm$ SEM. N.S.: Not significant, Mann
575 Whitney test. (C) Time schedule for the injection of α -syn PFFs and drug treatment. In Fig.
576 4, “PER (pre)” refers to “the mice in which PER treatment was initiated prior to the injection
577 of α -syn PFFs,” whereas “PER (post)” refers to “the mice in which PER treatment was
578 initiated after the injection of α -syn PFFs.” (D) Representative images of
579 immunohistochemical staining of the mice that underwent injection of α -syn PFFs and drug
580 treatment. Insets show high-power images of p- α -syn-positive pathology in the AON and
581 PC. Data are representative of two independent experiments. Scale bar: 200 μ m; inset: 50
582 μ m. (E) Area of p- α -syn-positive pathology in the AON and PC. Plotted data are pooled
583 from two independent experiments ($n = 6$ –8). Data are normalized against control and are
584 expressed as mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, N.S.: Not significant, Kruskal-Wallis test
585 with Dunn’s *post hoc* test. (F) Numbers of neuronal p- α -syn-positive aggregates in the
586 AON and PC. Plotted data are pooled from two independent experiments ($n = 6$ –8). Data
587 are normalized against control and are expressed as mean $\pm$ SEM. $**P < 0.01$, N.S.: Not

588 significant, Kruskal-Wallis test with Dunn's *post hoc* test. (G) Level of p- α -syn in the Triton
589 X-insoluble fraction. The numbers (in kDa) to the right indicate the position of the size
590 markers. Representative images and plotted data are shown (n = 5). All values are
591 expressed as mean $\pm$ SEM. **P* < 0.05, Kruskal-Wallis test with Dunn's *post hoc* test.

592

593 **Supporting Information FIG. S1**

594 Most of the cells colocalized with pHrodo-PFFs are NeuN-positive cells. (A) Numbers of
595 NeuN or GFAP-positive cells per field. Plotted data are representative of three
596 independent experiments (n = 8). Data are expressed as mean $\pm$ SEM. (B) Numbers of
597 NeuN or GFAP-positive cells per field colocalized with pHrodo-PFFs. Plotted data are
598 representative of three independent experiments (n = 8). Data are expressed as mean $\pm$
599 SEM.

600

601 **Supporting Information FIG. S2**

602 NBQX and TTX show no toxicity to primary neuronal culture. (A) (B) Density of neurons.
603 Data are representative of three independent experiments (n = 6). Data are normalized
604 against control and are expressed as mean $\pm$ SEM. N.S.: Not significant, Mann Whitney

605 test. Scatter plots show data from each sample. (C) (D) LDH assay. Plotted data are
606 representative of three independent experiments (n = 6). Data are normalized against
607 control and are expressed as mean $\pm$ SEM. N.S.: Not significant, Mann Whitney test.

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622 Financial disclosure of all authors (for the preceding 12 months)

623 J.U. and T.T. have no financial disclosure. N.U. received Japan Society for the

624 Promotion of Science (JSPS) Overseas Research Fellowships. M.S. received grants from

625 JSPS and Sumitomo Dainippon Pharma Co., Ltd. M.I. received a grant from JSPS. S.K.

626 received a grant from JSPS. Y.T. is a principal investigator of clinical trials by Kyowa Kirin

627 Co., Ltd., and Kissei Pharmaceutical Co., Ltd., and received a grant from JSPS. S.M.

628 received grants from JSR Co., Ltd, ROHTO Pharmaceutical Co., Ltd, and NOASTEC

629 Foundation. H.Y. received grants from JSPS. R.T. received consultancies from KAN

630 Research Institute, Inc., Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd.;

631 grants/research support from Sumitomo Dainippon Pharma Co., Ltd., Takeda

632 Pharmaceutical Co., Ltd., Eisai Co., Ltd., Kyowa Kirin Co., Ltd., Sanofi K.K., Otsuka

633 Pharmaceutical Co., Ltd., and Nippon Boehringer Ingelheim Co., Ltd.; grants from Japan

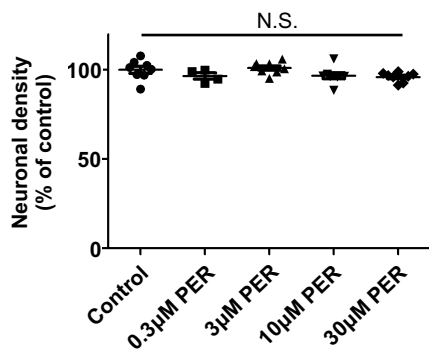
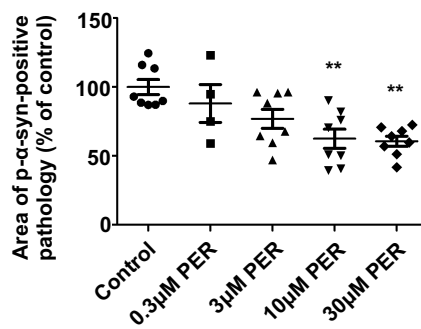
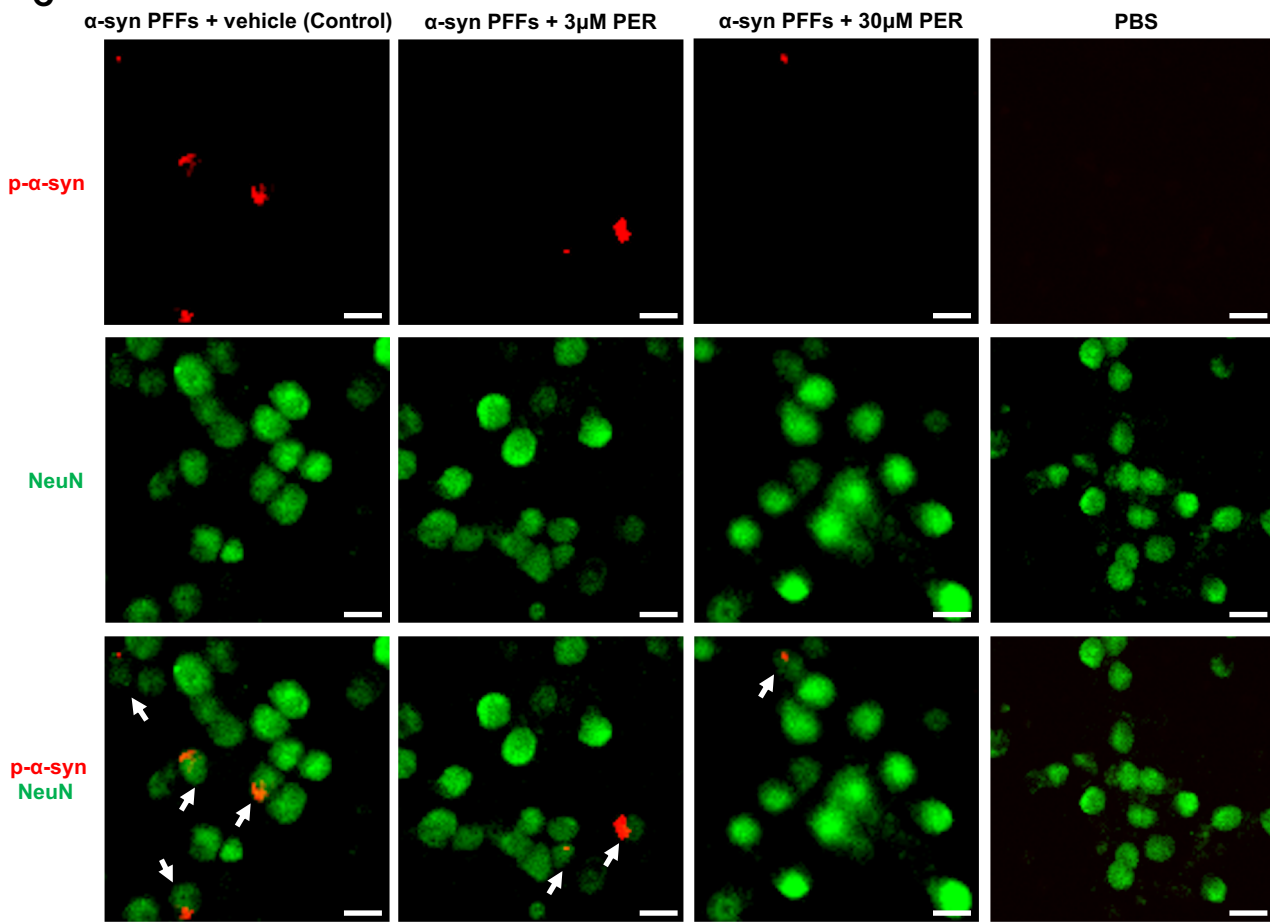
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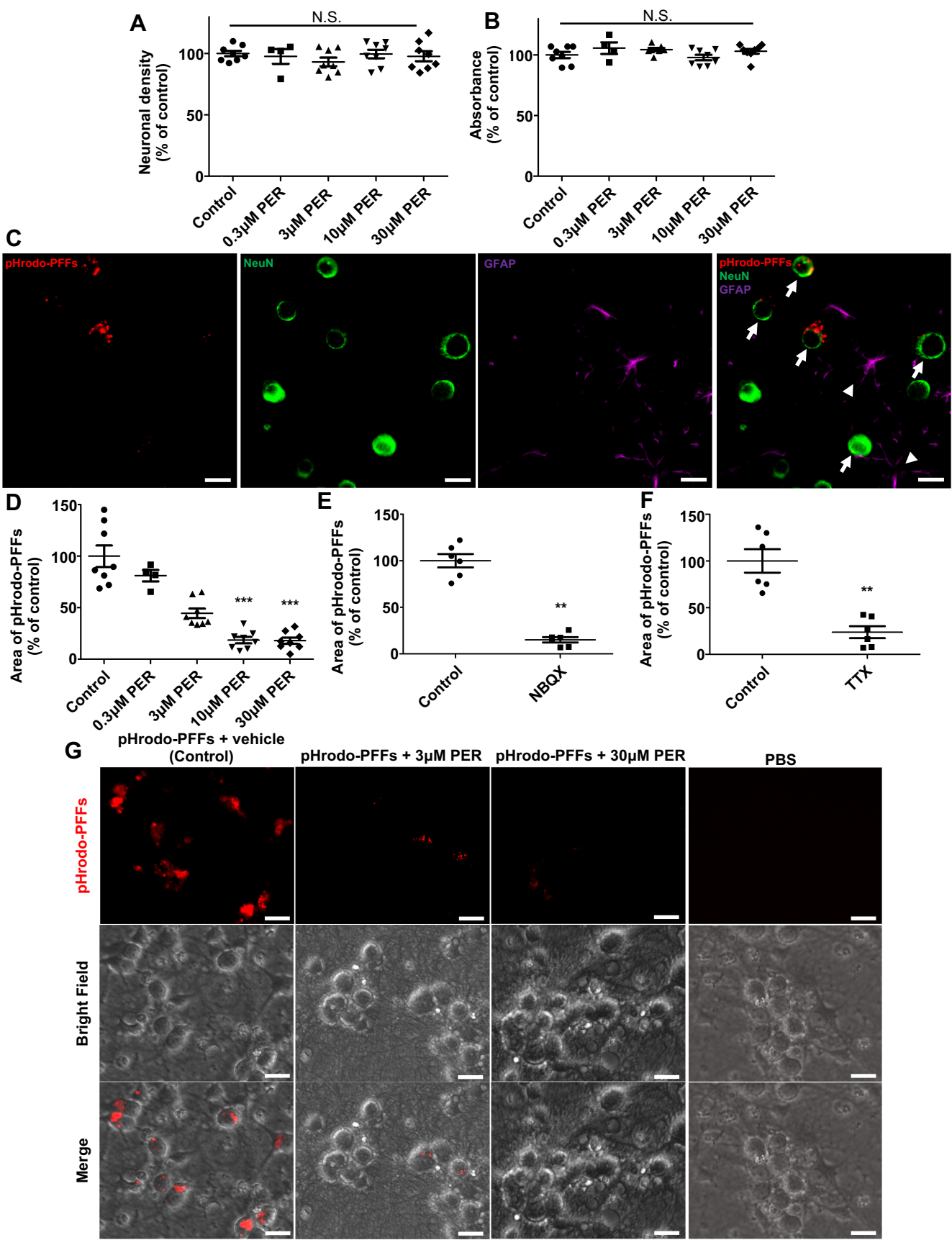
635 Culture, Sports, Science and Technology Japan; and honoraria from Sumitomo Dainippon

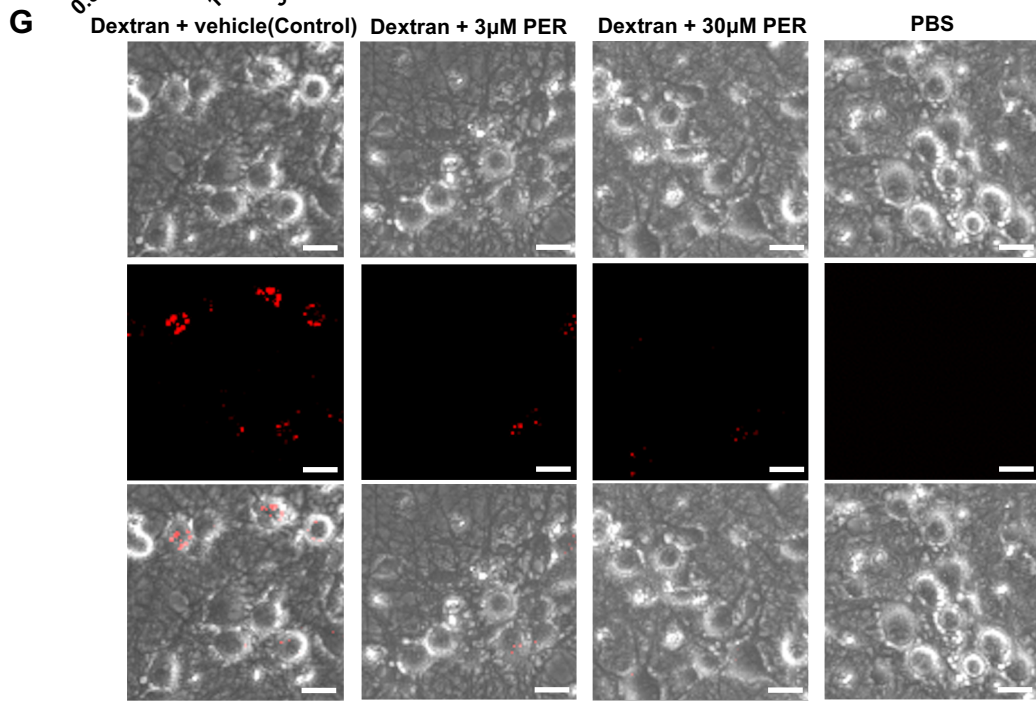
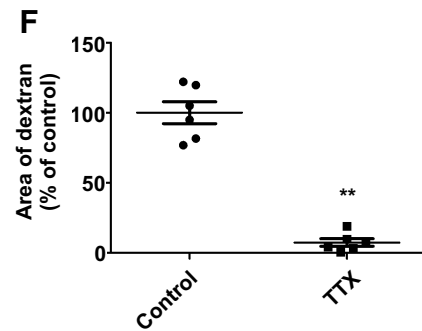
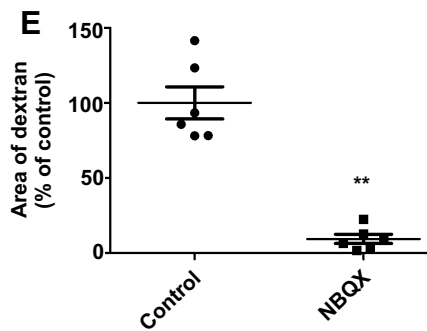
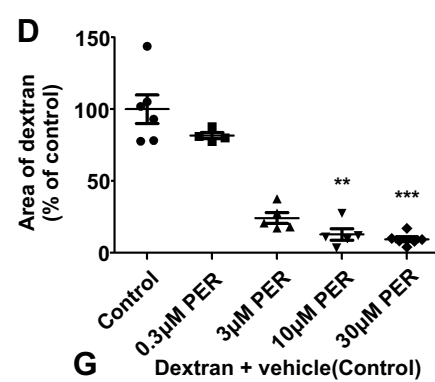
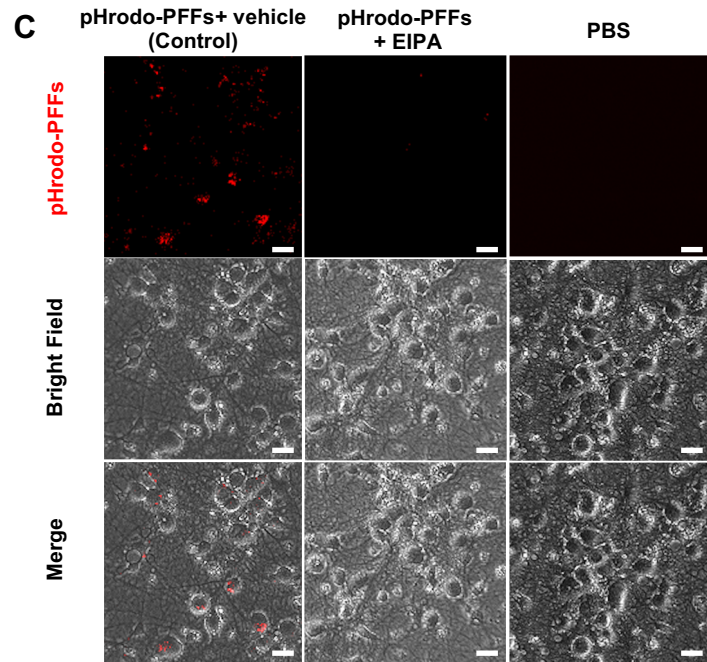
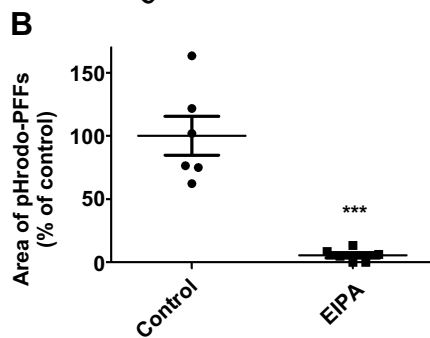
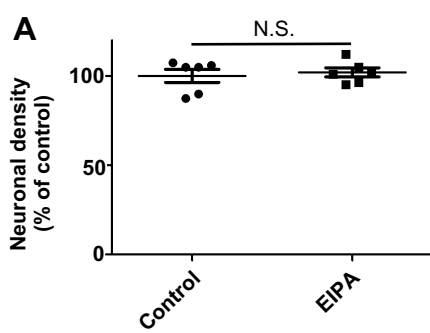
636 Pharma Co., Ltd., Takeda Pharmaceutical Co., Ltd., Novartis Pharma K.K., Kyowa Kirin

637 Co., Ltd., Eisai Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Ono Pharmaceutical Co., Ltd.,

638 AbbVie Inc, and Alexion Pharmaceuticals, Inc.

A**B****C**





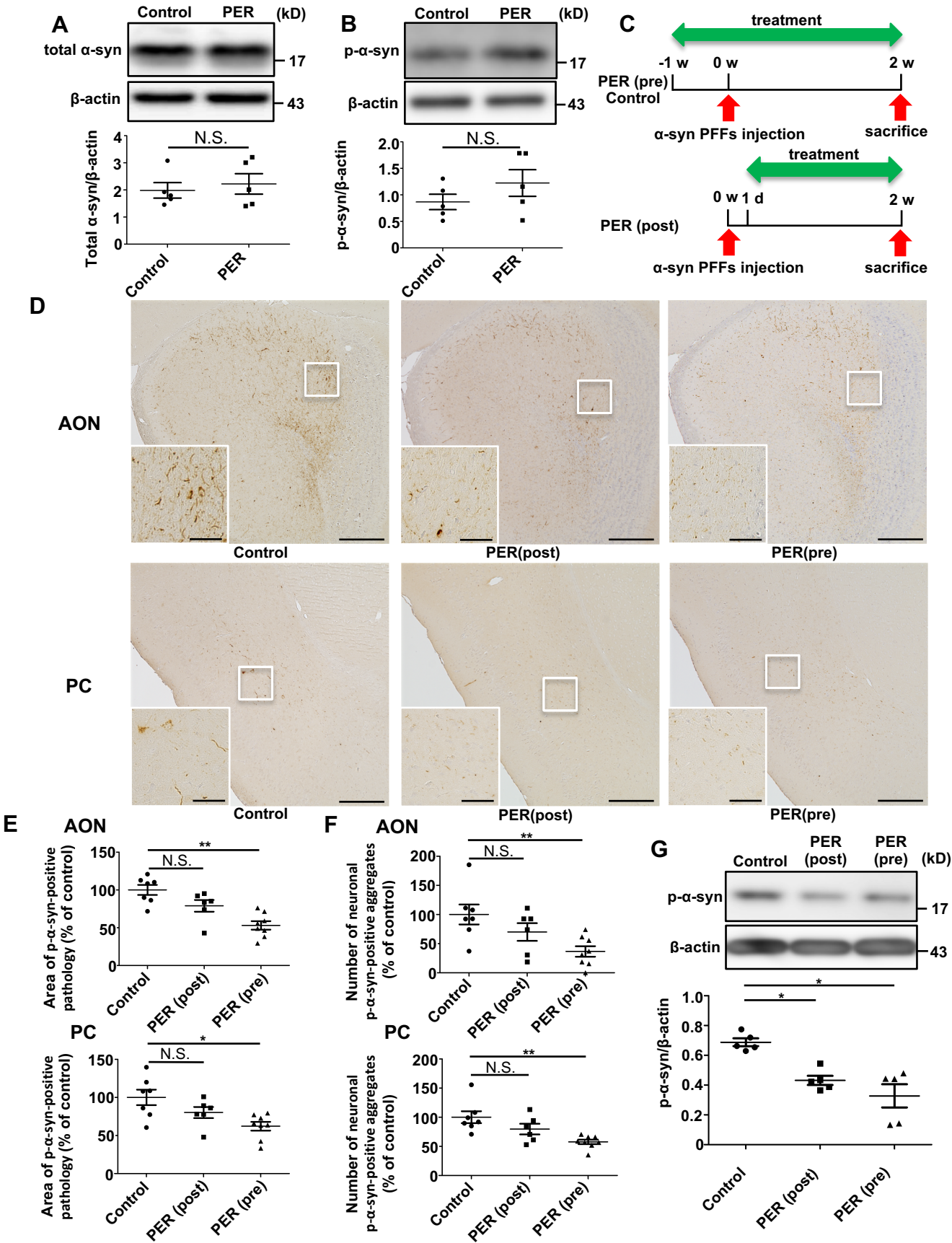
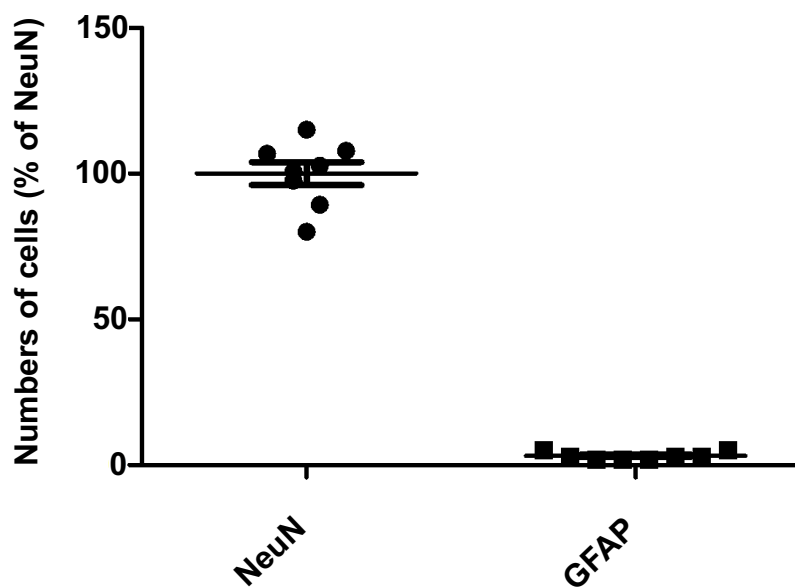


Fig.S1

A



B

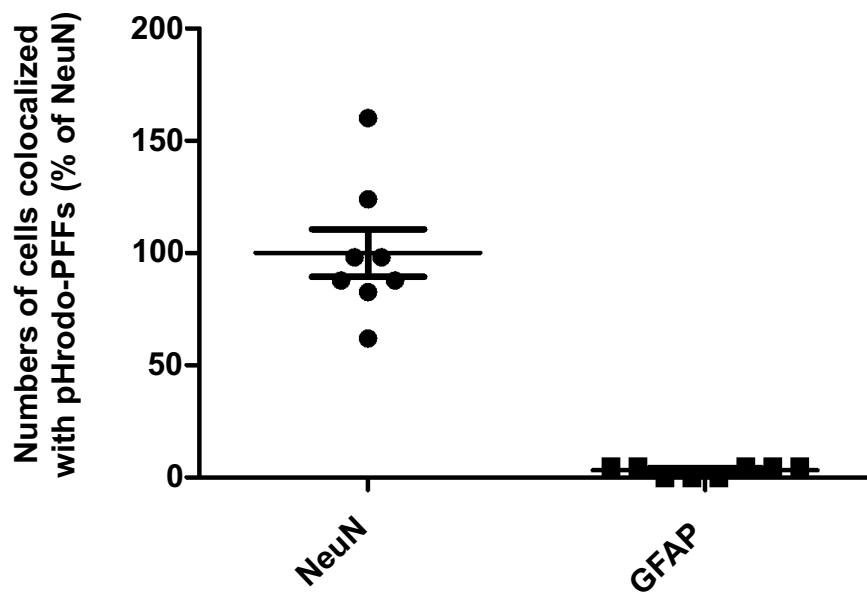


Fig.S2

