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► To cite this version:

Clara Grudina, Georgia Kouroupi, Takashi Nonaka, Masato Hasegawa, Rebecca Matsas, et al.. Human NPCs can degrade α -syn fibrils and transfer them preferentially in a cell contact-dependent manner possibly through TNT-like structures. *Neurobiology of Disease*, 2019, 132, pp.104609. 10.1016/j.nbd.2019.104609 . pasteur-02941667

HAL Id: pasteur-02941667

<https://pasteur.hal.science/pasteur-02941667v1>

Submitted on 20 Jul 2022

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1 ***Human NPCs can degrade α -syn fibrils and transfer them***
2 ***preferentially in a cell contact-dependent manner possibly***
3 ***through TNT-like structures***

4
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16

17 **Highlights**

- 18 • α -syn fibrils are internalized by hNPCs and directed to lysosomes.
19 • α -syn fibrils are degraded over time by hNPCs.
20 • α -syn fibrils can propagate between hNPCs preferentially in a cell contact
21 dependent manner possibly in TNT-like structures.

22

23 **Abstract**

24 Parkinson's disease (PD) is the second most common neurodegenerative disorder
25 whereby loss of midbrain dopaminergic neurons results in motor dysfunction.

26 Transplantation of human induced pluripotent stem cells (iPSCs) into the brain of
27 patients affected by PD is one of the therapeutic approaches that has gained interest
28 to compensate for the degeneration of neurons and improve disease symptoms.
29 However, only a part of transplanted cells can differentiate into mature neurons while
30 the majority remains in undifferentiated state. Here we investigated whether human
31 neuronal precursor cells (hNPCs) derived from iPSCs have an active role in α -
32 synuclein (α -syn) pathology. Our findings demonstrate that α -syn fibrils are taken up
33 by hNPCs and are preferentially localized in lysosomes where they can be degraded.
34 However, α -syn fibrils are also transferred between hNPCs in a cell-to-cell contact
35 dependent manner, and are found in tunneling nanotube (TNT)-like structures. Thus,
36 NPCs can have a dual role in the progression of α -syn pathology, which should be
37 considered in human transplants.

38

39 **Keywords**

40 Parkinson's disease; human neuronal precursors; alpha-synuclein; lysosomes; TNT-
41 like structures

42

43 **Abbreviations**

44 PD, Parkinson's disease; α -syn, α -synuclein; iPSCs, induced pluripotent stem cells;
45 hNPCs, human neuronal precursor cells; TNTs, tunneling nanotubes; TH, tyrosine
46 hydroxylase.

47

48 **1.Introduction**

49 α -Synuclein (α -syn) is a presynaptic protein that plays a central role in the
50 pathogenesis of a group of neurodegenerative diseases defined as

51 synucleinopathies, including Parkinson disease (PD). Intracellular deposits of
52 aggregated α -syn within the neuron's soma and neurites, respectively known as
53 Lewy bodies and Lewy neurites (Braak et al., 1999), are key features in these
54 pathologies. Despite progress, the physiological functions of α -syn are still unclear.
55 Several studies have shown that α -syn is involved in compartmentalization, storage,
56 and recycling of neurotransmitters (Allen Reish and Standaert, 2015). A key feature
57 for the pathological role of this protein is that it exists in different conformations,
58 including monomeric and oligomeric states, that promote or impede its aggregation
59 (Conway et al., 1998; Karpinar et al., 2009; Nuber et al., 2018). Furthermore different
60 mutations of the protein, such as p.A53T cause autosomal dominant forms of PD
61 (Tan et al., 2005) and can strongly promote and accelerate α -syn aggregation.

62 The cellular mechanisms underlying the initiation and propagation of α -syn pathology
63 are still under investigation. Previous studies have shown that α -syn aggregates can
64 spread in a prion-like manner from cell-to-cell in the brain *in vitro* as well as *in vivo*
65 (Desplats et al., 2009; Freundt et al., 2012; Sacino et al., 2014; Peelaerts et al.,
66 2015; Abounit et al., 2016; Brundin and Melki, 2017). Particularly, we have reported
67 that mouse neuronal cells and primary neurons efficiently internalize fluorescent α -
68 syn fibrils, direct them to lysosomal vesicles and transfer them to other neurons
69 inside lysosomes in a contact-dependent manner through TNTs (Abounit et al., 2016).
70 Moreover, we have shown that astrocytes can internalize α -syn fibrils and transfer
71 them efficiently to astrocytes but not to neurons, indicating that the capacity to
72 transfer fibrils can be cell-dependent. However differently from neurons, astrocytes
73 are able to efficiently degrade fibrillar α -syn, suggesting an active role for these cells
74 in clearing α -syn deposits (Loria et al., 2017).

75 Until now, no therapy is available to hold up or at least slow down the progress of
76 neurodegeneration in the brain of PD patients. One approach that has gained
77 considerable attention is the development of cell-based therapies to compensate
78 dopaminergic neuronal loss and dopamine deprivation with new healthy neurons
79 inducing major, long-lasting improvement. Although it is still unclear whether
80 dopaminergic neuron degeneration is an initial feature of the disease or the
81 unavoidable result of multiple dysfunctions throughout the brain, it represents a
82 common pathological manifestation in PD and is responsible for many of the clinical
83 symptoms, including motor dysfunction. Therefore several cellular sources have
84 been considered for transplantation, including human induced pluripotent stem cells
85 (iPSCs). However, a crucial aspect in this approach is that the transplanted cells
86 must survive for a long time, differentiate to the appropriate neuronal phenotype and
87 finally integrate into the host tissue. Recent studies have demonstrated that 6-54% of
88 the transplanted cells survive and express tyrosine hydroxylase (TH) at 6–18 weeks
89 in a rat model (Freed et al., 2001; Kriks et al., 2011) whilst only $33.3 \pm 24.4\%$ become
90 TH positive at 6 months in a primate model (Kikuchi et al., 2011, 2017). Accordingly,
91 many neuronal precursor cells have been found in the graft suggesting that an
92 important part of these transplanted cells may not be able to differentiate into
93 dopaminergic neurons. A pertinent question is therefore whether α -syn pathology can
94 be transferred from the tissue to such neuronal precursors that may further
95 propagate the disease.

96 In the present study, we used human iPS cell-derived neuronal precursors (hNPCs)
97 differentiated towards the dopaminergic lineage to investigate whether these cells
98 could have an active role in the internalization and propagation of α -syn fibrils. We
99 demonstrate that α -syn fibrils are internalized by hNPCs and are localized in

100 lysosomal vesicles where they are subjected to degradation. Further, we observed
101 that α -syn fibrils can be transferred between hNPCs, primarily by cell-cell contact and
102 to a much lesser extent through a secretory route. In particular, we showed that
103 hNPCs are able to form TNT-like structures (Sartori-Rupp et al., 2019) and that α -syn
104 fibrils can be found inside these structures formed between two different population
105 of hNPCs.

106 Overall, our *in vitro* study reveals that hNPCs are capable of fibrillary α -syn uptake,
107 intercellular transfer and degradation and may play a role in modulating α -syn
108 pathology.

109

110 **2. Materials and Methods**

111

112 **2.1 Culture of human iPSCs and differentiation to dopaminergic neuronal** 113 **precursor cells (NPCs)**

114 WT and A53T-iPSC lines were generated and characterized as previously described
115 (Kouroupi et al., 2017). iPSCs were grown in pre-coating Geltrex (Life Technologies)
116 plates in medium TeSR™-E8™ (StemCell Technologies). ReLeSR (StemCell
117 Technologies) was used to passage iPSCs weekly. WT and A53T α -syn iPSCs were
118 dissociated with EDTA 0,5mM for 2 min at 37°C and re-suspended in falcons with
119 iPSC medium TeSR™-E8™ (StemCell Technologies). iPSC clumps were allowed to
120 sink for ten minutes, were then resuspended in embryoid body (EBs) medium (1:1
121 DMEM/F12 and Neurobasal medium, 1x P/S, 1x N2, 2x B27 without vitamin A, 100
122 μ M β Mercaptoethanol) supplemented with 10 μ M Rock Inhibitor Y-27632 (Tebu),
123 10 μ M SB431542 (Tebu), 100nM LDN193189 (Tebu) and plated in Nunc no-treated
124 flasks for suspension cell cultures (ThermoFisher Scientific). Medium was changed

125 every day until day 8 when EBs were plated onto p60 plates pre-coated with poly-L-
126 ornithine 20µg/mL (Life Technologies) and laminin 10µg/mL (Sigma). The medium
127 was then changed to NPC medium (Neurobasal A, 1x P/S, 1x N2, 2x B27 without
128 vitamin A, 100 µM βMercaptoethanol, 1x Glutamax, supplemented with 200ng/ml
129 SHH (R&d Systems), 100ng/ml FGF-8b (R&d Systems), 20ng/ml bFGF (Life
130 Technologies), 20ng/ml EGF (Life Technologies) until day 12. At day 13 STEMdiff™
131 Neural Rosette Selection Reagent (StemCell Technologies) was used to select
132 neural rosettes by micropipette. Rosettes were plated onto p60 plates pre-coated
133 with poly-L-ornithine 20µg/mL (Life Technologies) and laminin 10µg/mL (Sigma) and
134 were grown in NPC medium supplemented with only 20ng/ml bFGF (Life
135 Technologies), passage p0. For next passages accutase (Sigma) was used. All
136 experiments were performed using NPCs from p3 to p6 grown in NPC medium.
137 All procedures for generating iPSC-NPCs were approved by Comité de Recherche
138 Clinique, 825 Institut Pasteur, Paris (approval number 2015-034).

139

140 **2.2 Expression, purification, preparation and labelling of alpha-syn fibrils**

141 Human wild-type α-syn in pRK172, a construct containing α-syn that lacks cysteine
142 because of mutagenesis of codon 136 (TAC to TAT) as described previously
143 (Masuda et al., 2006), were transformed into *Escherichia coli* BL21 (DE3).
144 Expression and purification were performed as described previously (Nonaka et al.,
145 2005, 2010). The protein concentrations of monomeric α-syn were determined by
146 RP-HPLC as described previously (Nonaka et al., 2005, 2010). Purified recombinant
147 α-syn monomers (~5 mg/ml) containing 30 mM Tris-HCl, pH 7.5, 10 mM DTT, and
148 0.1% sodium azide were incubated at 37 °C with shaking using a horizontal shaker
149 (TAITEC) at 200 rpm. After incubation for 7 days, the samples were ultracentrifuged

150 at 100,000 g for 20 min at room temperature, and the ppt fraction was recovered as
151 α -syn fibrils. The samples were re-suspended in saline and ultracentrifuged again.
152 The resultant pellets were re-suspended in saline and sonicated with an ultrasonic
153 homogenizer (VP-5S, TAITEC). The fibrils were labelled with Alexa Fluor 568 Protein
154 Labeling Kit (Invitrogen) according to the manufacturer's instructions. After incubation
155 with Alexa Fluor dye, the samples were ultracentrifuged again. The pellets were re-
156 suspended in 30 mM Tris-HCl, pH 7.5 and ultracentrifuged again. The labelled α -syn
157 fibrils were re-suspended in saline containing 0.1% sodium azide. The protein
158 concentration of the fibrils was determined by RP-HPLC as described previously
159 (Nonaka et al., 2005, 2010). To check the *in vitro* seeding activity of the labelled
160 fibrils, the fibrils (3 μ g) were added to 100 μ L of 1 mg/mL α -syn monomer in 30 μ M
161 Thioflavin T and 80 mM Hepes, pH 7.5. Amyloid-like fibril formation was continuously
162 monitored in terms of thioflavin T fluorescence (excitation 442 nm, emission 485 nm)
163 with a plate reader (Varioskan Flash, Thermo Scientific).

164

165 **2.3 Internalization assay of α -syn fibrils in hNPCs**

166 hNPCs derived as described above were treated with 0.3 μ M of Alexa- 568
167 fluorescent-tagged human recombinant α -syn fibrils. Fibrils were diluted in the
168 appropriate medium for each culture and sonicated for 5 min at 80% amplitude with a
169 pulse cycle of 5s on and 2s off in an ultrasonic water bath Vibra-Cell 75041
170 (BioBlocks Scientific). Internalization was assessed at different time points after cells
171 were washed using trypsin diluted 1:3 in PBS (3 times) to prevent the fibrils from
172 remaining attached to the membrane, and finally culture medium was replaced (Fig.
173 S2). All samples were fixed and then labeled with far red Wheat Germ Agglutinin
174 (WGA) (Thermo Fisher) to visualize the plasma membrane.

175

176 **2.4 Co-culture systems of hNPCs**

177 In order to obtain two different cell populations, hNPCs donor cells were treated with
178 0.3 μ M of Alexa- 568 α -syn sonicated fibrils overnight. The following day, donor cells
179 were washed using trypsin diluted 1:3 in PBS (3 times) to remove eventual
180 fibrils that might have attached to the plasma membrane, detached by Accutase
181 (Sigma), counted and mixed (ratio1: 1) in suspension with the acceptor cells (hNPCs
182 transfected by LV-GFP or labeled with CellMask Green (Invitrogen)), and co-cultured
183 for 24h. Then, cells were fixed and immunostained. For quantification of α -syn
184 transfer at least 100 cells were analysed in each independent experiment (n=3).

185

186 **2.5 Secretion experiment**

187 Donor cells were treated with 0.3 μ M of Alexa- 568 α -syn sonicated fibrils overnight.
188 The following day, the medium was removed and replaced with fresh for 24h. The
189 next day, the conditioned medium was added to acceptor cells (hNPCs transfected
190 by LV-GFP or labeled with CellMask Green (Invitrogen)) for 24h. After, cells were
191 fixed, stained and analysed. For quantification of α -syn transfer at least 100 cells
192 were analysed in each independent experiment (n=3).

193

194 **2.6 Cell viability assay by measuring LDH release**

195 Three independent experiments were performed following the manual instruction of
196 Cytotoxicity Detection Kit (LDH) (Roche). To measure the absorbance of the samples
197 96-well plate (Falcon) was read using Infinite® M200 PRO Tecan Fluorescence
198 Microplate Reader at 492nm.

199

200 **2.7 Western Blot**

201 hNPCs cultures were lysed using RIPA buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl,
202 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate
203 (SDS), 1mM sodium orthovanadate, 1 mM NaF) containing protease inhibitor tablet
204 (Roche), centrifuged for 15min at 3000 rpm. Protein concentration was estimated in
205 the supernatant by Bradford assay (Biorad). Samples were subjected to 4-15% Mini
206 Protean TGX Stain-Free gels (Biorad) and transferred onto 0.45µm pore size
207 nitrocellulose membrane (Biorad). Non-specific binding sites were blocked in TBS
208 0.1% Tween 20/ 5% milk for 1 hour at RT followed by overnight incubation with
209 mouse anti-α-synuclein (1:1000; BD Biosciences BD610787) or mouse anti-α Tubulin
210 (1:2000; Sigma T9026) or rabbit anti-GAPDH (1:2000; Santa Cruz sc-25778).
211 Incubation with appropriate HRP-conjugated secondary antibodies (GE Healthcare)
212 was performed for 2 hours at RT and protein bands were visualized using Amersham
213 ECL Prime Western Blotting Detection Reagent (GE Healthcare). The experiment
214 was performed 3 times.

215

216 **2.8 Immunocytochemistry of hNPCs**

217 Immunostaining of hNPCs was performed following the same protocol. Cells were
218 rinsed with PBS and fixed with 4% paraformaldehyde for 15min at 4°C, then cells
219 were permeabilized and blocked with blocking solution (PBS 0.1% Triton, 5% FBS)
220 for 1h in blocking solution. For only LAMTOR4 antibody cells were permeabilized 2
221 minutes with PBS 0.1% Triton and then blocked with PBS 10% BSA. Primary
222 antibodies were incubated overnight at 4°C diluted in appropriate blocking solution.
223 After rinsing with PBS, cells were incubated with Alexa-conjugated secondary
224 antibody for 1h at room temperature (dilution 1:600), and nuclei were counterstained

225 with DAPI (1:1000; Sigma Aldrich). Coverslips were mounted using aqua-poly/mount
226 (Polysciences). The antibodies used: rabbit anti-Nestin (1:200; Merck Millipore
227 ABD69), mouse anti-PAX6 (1:50; Developmental Studies Hybridoma Bank), goat
228 anti-Doublecortin (DCX, 1:100; Santa Cruz sc-8067), rabbit anti-Glial fibrillary acidic
229 protein (GFAP, 1:500; DAKO Z0334), rabbit anti-LAMTOR4 (1 :1000 ; Cell Signaling
230 12284), mouse anti- α -synuclein Syn-1 (1:500; BD Biosciences BD610787), rabbit
231 anti-LMX1 (1:1000 ; Merck Millipore AB10533), rabbit anti-SOX2 (1 :750 ; Abcam
232 ab59776), rabbit anti-FoX2a (1:400 ; Abcam ab108422), chicken anti-MAP-2 (1:500;
233 Merck Millipore AB15452), rabbit anti-TH (1 :500 ; Merck Millipore AB152).

234 For TNTs images cells were fixed with fixative solution 1 for 15 min (2%PFA, 0.05%
235 glutaraldehyde and 0.2 M HEPES in PBS) and then with fixative solution 2 for
236 another 15 min (4% PFA and 0.2 M HEPES in PBS).

237

238 **2.9 Lysotracker Staining**

239 To visualize lysosomes during co-culture and secretion experiment, donor and
240 acceptor cells were labelled using Lysotracker Deep Red (Thermo Fisher Scientific
241 L12492). Cells are incubated with the dye diluted 10nM in growth medium for 30
242 minutes at 37°C. After, the cells are rinsed 3 times with PBS and fixed with 4% PFA.

243

244 **2.10 Genomic DNA analysis for detection of the p.A53T (G209A) mutation**

245 Genomic DNA of iPSCs was extracted by Quick-DNA Microprep Kit (Zymo
246 Research) following the manufacturer's recommended protocol. Specific primers
247 were used for SNCA gene (Forward: GCTAATCAGCAATTTAAGGCTAG, Reverse:
248 GATATGTTCTTAGATGCTCAG) and PCR products were digested by the restriction

249 enzyme Tsp45I (New England Biolabs). p.A53T (G209A) mutation results in a novel
250 Tsp45I site and two additional fragments of 128 and 88 bp can be detected.

251

252 **2.11 Acquisition and analysis of immunostained images at optical confocal** 253 **microscopy**

254 Images were acquired with an inverted laser scanning confocal microscope LSM700
255 (Zeiss), with a 63x objective (zoom 0.7 or 1). Images were acquired using the Zen
256 acquisition software (Zeiss) and further processed with ICY software (Quantitative
257 Image Analysis Unit, Institut Pasteur <http://icy.bioimageanalysis.org/>). In all
258 experiments, it was acquired Z-stacks covering the whole volume of cells.

259 In details for the transfer experiments, in order to quantify the percentage of donor
260 and acceptor cells containing α -syn puncta, the Z-stack was divided into the lower
261 and upper part, segmenting only donor or acceptor cells, when possible, and then
262 projecting the maximum intensity of those slices, using the ICY software. This was
263 done in order to only have the whole donor or acceptor cell volume and to focus on
264 what was inside the cells. Quantification of images was performed manually scrolling
265 through the slices of the Z-stack to identify the puncta that were located inside the
266 cell body based on nucleus identification and proximity. Overlapping cells were
267 excluded from the analysis. Whereas all the images showed in the figures of hNPCs
268 alone are projections of the entire Z- stack, the orthogonal views and images showed
269 of co-culture experiments correspond to projections of selected slices of the Z-stack.
270 For co-localization analysis all images were acquired using the same parameters at
271 confocal microscope. At least 50 single cells from different images were analysed by
272 JaCoP plugin in ImageJ. The Pearson correlation coefficient was used to quantify the

273 degree of colocalization between fluorophores α -syn fibrils puncta and lysosomes
274 labelled by LAMTOR4 antibody.

275 Analysis was performed in 3 independent experiments.

276

277 **2.12 Statistical analysis**

278 Statistical analyses and graphs were performed using the GraphPad Prism version 6
279 software. All the results are expressed as the mean \pm s.e.m. For comparisons
280 between two groups the Mann-Whitney test was used. Unless stated in the figure's
281 legend, for comparisons between more than two groups, one-way ANOVA with
282 Tukey's post hoc test was employed. In all cases, statistical significance was
283 attributed when $p \leq 0.05$.

284

285 **3. Results**

286 **3.1 Internalization of α -syn fibrils in hNPCs**

287 To generate hNPCs, iPSCs produced from a healthy donor (Kouroupi et al., 2017)
288 were differentiated to the dopaminergic lineage following a dual SMAD inhibition
289 protocol (Chambers et al., 2009) as described in Materials and Methods section. For
290 comparison, iPSCs generated from a patient carrying the G209A (p.A53T) mutation
291 in the α -syn gene (Kouroupi et al., 2017) were also differentiated to hNPCs. After 25
292 days of differentiation, a committed population of neuronal precursor cells with
293 dopaminergic (DA) identity was obtained. In particular, approximately 80% of cells
294 were Sox2⁺/Nestin⁺ neuronal precursors, while co-staining with Nestin and Pax6 has
295 shown that 51% of Nestin⁺ cells were Pax6⁺ committed neuronal precursors (39% in
296 the total population) (Fig.S1A). GFAP⁺ cells comprised only 1.6% of the total cell
297 population whilst the remaining cells (17.9% of the total population) were DCX⁺ early

298 born neurons. hNPCs were also assessed for expression of dopaminergic lineage
299 markers showing that □31% were Lmx1⁺ and □25% Fox2a⁺ (Fig.S1A) . Finally, they
300 were tested for their ability to differentiate *in vitro* into neurons, a fraction of which
301 already expressed TH (□18% of cells positive) at 30 days (Fig.S1C).

302 We first examined whether recombinant Alexa 568 labeled human α -syn fibrils were
303 capable of entering hNPCs. Time course experiments (Fig.1A, B) based on confocal
304 images acquisition at different time points, revealed that α -syn fibrils were rapidly
305 internalized with high efficiency. After 1h following α -syn fibril loading, the percentage
306 of hNPCs containing fluorescent puncta was already around 80%, reaching 98%
307 after 16h (Fig.1B). No differences between the two genotypes (WT and A53T-syn)
308 were observed suggesting that the mutation does not impair internalization of α -syn
309 fibrils. Then, we used a specific ICY software script to automatically detect and
310 quantify the number and size of α -syn fibril puncta at different time points (Fig.1C).
311 By this method, we found that both the average number and size of α -syn fibril
312 puncta per cell did not significantly change from 3h to 16h, showing no significant
313 difference between the two genotypes (Fig.1C).

314 These results indicate that hNPCs (WT and A53T-syn) are capable of uptaking α -syn
315 fibrils very efficiently. We next analysed if the exposure to α -syn fibrils could generate
316 a toxic effect in hNPCs.

317

318 **3.2 Lactate dehydrogenase (LDH) release after exposure to α -syn fibrils in** 319 **hNPCs**

320 To investigate if α -syn fibrils could have toxic effects in hNPCs, we performed time
321 course measurements of lactate dehydrogenase (LDH) release in the absence
322 (control) and after exposure to α -syn fibrils (0.3 μ M) (Fig.2). The results showed that

323 there was an increase in LDH release during the time of cell culture, as expected, but
324 no significant increase was observed between control cells and cells (from both
325 genotypes) exposed to α -syn fibrils. Thus, we concluded that α -syn fibrils were not
326 toxic within the timeframe of our experiments (Fig.2) and the two genotypes did not
327 show differences in terms of cell viability.

328

329 **3.3 α -Syn fibrils are found in lysosomal vesicles in the cytosol of hNPCs**

330 Previous studies showed that α -syn fibrils taken up from the medium are
331 preferentially directed to the lysosomal compartment for degradation in different cell
332 types (Hasegawa et al., 2011; Konno et al., 2012; Lee et al., 2005, 2008; Sung et al.,
333 2001), thus next we analyzed the intracellular localization of α -syn fibrils following
334 uptake by hNPCs. We found that also in hNPCs α -syn fibril puncta co-localize with
335 lysosomal vesicles (Fig.3). We quantified the co-localization between fluorescent α -
336 syn fibril puncta and lysosomes by using LAMTOR4 (a lysosomal marker)
337 immunofluorescence (Pu et al., 2017). Taking into consideration the Pearson
338 correlation coefficient (by JaCoP plugin, ImageJ, see material and methods), we
339 found that after only 3h following α -syn fibril loading, α -syn fibrils were already inside
340 lysosomes. Specifically, the percentages of co-localization during the experimental
341 time frame (from 3h to 16h) were 52.8 ± 0.057 ; 58.5 ± 0.096 ; 62 ± 0.04 ; 45 ± 0.043 in
342 WT hNPCs and 49 ± 0.04 ; 56 ± 0.03 ; 56 ± 0.043 ; 47 ± 0.02 in A53T hNPCs (Fig.3A).
343 The fibrils persisted in lysosomes without significant differences in terms of their co-
344 localization even after 16h. Moreover, the subcellular localization did not show any
345 significant differences between the two genotypes.
346 These results suggest that also in hNPCs, fibrils are directed to the lysosomal
347 compartment and the A53T mutation does not affect this process.

348

349 **3.4 α -Syn fibrils are degraded rapidly in hNPCs**

350 We have previously shown that the ability of lysosomes to degrade up-taken α -syn
351 fibrils is cell type dependent (Loria et al., 2017). To address if α -syn fibrils stored in
352 lysosome compartments can be degraded by hNPCs, we quantified the total amount
353 of α -syn by Western Blot at different time points. hNPCs were loaded, or not (control
354 sample), with α -syn fibrils for 16h, then the cells were washed, kept in culture and
355 lysed at 16h, 1 day and 3 days. As previously reported (Bayer et al., 1999; Galvin et
356 al., 2001; Raghavan et al., 2004), endogenous α -syn was detected at low levels in
357 control hNPCs and increased considerably upon 16h exposure to α -syn fibrils.
358 Surprisingly, we observed that in both genotypes α -syn fibrils were degraded very
359 efficiently. In particular, significant degradation was observed between 1 day and 3
360 days (Fig.4A, B).

361 To confirm that this degradation was correlated to lysosome activity, we performed
362 the same experiment described above, adding for 36h two inhibitors of lysosomal
363 proteases: E64D (a membrane-permeable inhibitor of cathepsins B, H, and L) plus
364 pepstatin A (an inhibitor of cathepsins D and E)(Li et al., 2013; Yang et al., 2013) in
365 order to block degradation and visualize accumulation of fibrils inside the cells.
366 Indeed, after treatment with E64D (20 μ M) and pepstatin A (20 μ M) (E+P), we could
367 monitor by WB the accumulation of α -syn fibrils as compared with control (sample
368 without inhibitors lysed at the same time) (Fig. 4C). Qualitative immunofluorescence
369 experiments confirmed that the treatment with the two inhibitors induced the
370 accumulation of fibrils into hNPC; interestingly the pictures show that after 36H
371 treatment fibrils accumulate outside the lysosomes labeled by lysotracker (Fig. S2).

372 Overall this result demonstrated that hNPCs are able to degrade α -syn fibrils within 3
373 days following uptake and that this degradation occurs via the lysosomal pathway.

374

375 **3.5 α -Syn fibrils can be transferred between hNPCs**

376 In previous studies, we demonstrated that neuron-like CAD cells, primary murine
377 cortical neurons and murine astrocytes propagate α -syn fibrils to other cells, and the
378 transfer occurs preferentially inside lysosomal vesicles through tunneling nanotubes
379 (TNTs) (Abounit et al., 2016; Loria et al., 2017). In order to assess if hNPCs could
380 also have a role in the spreading of α -syn fibrils we tested their transfer ability in a co-
381 culture system. hNPCs were first loaded overnight with Alexa 568 labelled- α -syn
382 fibrils (donor cells). The following day donor cells were washed using trypsin diluted
383 1:3 in PBS (3 times) to remove eventual fibrils that might have attached to the
384 plasma membrane (Fig. S3), and co-cultured for 24h at a 1:1 ratio with hNPCs
385 expressing GFP by lentiviral transduction (LV-GFP) that allowed to distinguish
386 acceptor cells that were labeled in green (Fig.5A). Transfer of α -syn fibrils was
387 monitored by immunofluorescence and quantitative confocal microscopy. After 24h in
388 co-culture, we observed that both donor and acceptor cells contained α -syn puncta
389 (Fig.5B). By Z-stack imaging and orthogonal projection of cells we confirmed the
390 presence of α -syn puncta within the acceptor cells (Fig.5B). Using ICY software we
391 automatically detected and quantified the number of α -syn puncta. Under these
392 experimental conditions, we found that the average number of α -syn puncta inside
393 acceptor cells was 11.8 ± 1.31 in WT hNPCs and 15.34 ± 2.98 in A53T hNPCs
394 (Fig.5C) without significant differences in α -syn fibril transfer between genotypes.
395 Importantly, by using α -syn antibodies we could confirm that α -syn fibrils were
396 actually transferred in acceptor cells as we observed co-localization between α -syn

397 antibodies and Alexa 568 puncta (Fig.S4). These data indicated that hNPC were able
398 to efficiently transfer α -syn fibrils between them.

399 To understand whether this transfer occurred mainly through a cell to cell dependent
400 mechanism next we measured the amount of transfer occurring through the secretory
401 pathway. Specifically, we monitored whether α -syn fibrils could be secreted by
402 hNPCs in the medium and subsequently taken up from acceptor cells. To this aim,
403 hNPCs were first loaded overnight with Alexa 568 labelled- α -syn fibrils (donor cells),
404 then the medium was removed and replaced for 24h. Next, the conditioned medium
405 of 24 hours from the donor cells was added to acceptor cells (plated in different
406 dishes) for 24h (Fig.5D). We observed that α -syn fibrils were secreted by hNPCs and
407 uptaken by acceptor cells after 24h. However, the average number of puncta per
408 acceptor cell was low compared with the co-culture system (2.7 ± 0.6 in WT hNPCs
409 and 2.9 ± 0.5 in A53T hNPCs) (Fig.5E, F). Consistently, we also observed that the
410 percentage of acceptor cells containing α -syn puncta was lower in acceptor cells
411 exposed to conditioned medium compared with the co-culture conditions ($33.60 \pm$
412 7.00 vs 65.53 ± 4.165 in WT hNPCs and 32.75 ± 4.45 vs 57.29 ± 2.714 in A53T
413 hNPCs) (Fig.5 G). Altogether these data suggest that the amount of transfer through
414 a secretory pathway was less efficient. Moreover, we could show that hNPCs are
415 able to form numerous TNT-like-structures between themselves. These structures
416 contained α -syn puncta, suggesting that α -syn fibrils could be transferred through
417 these connections (Fig.5 H), similar to what was shown in neuronal cell lines (Abounit
418 et al., 2016).

419 Finally, we investigated the subcellular localization of α -syn puncta in acceptor cells
420 both after cell-to-cell mediated transfer and through secretion in the conditioned
421 medium. To this aim we used lysotracker to detect lysosomal vesicles in acceptor

422 cells. In both conditions α -syn puncta were found inside lysosomes in acceptor cells
423 similar to donor cells (Fig.S5); co-localization analysis between fibrils and lysotracker
424 did not show any significant differences between the two conditions (57 ± 0.02 in co-
425 culture and 59 ± 0.02 in secretion). This is consistent with previous data in neuronal
426 cells, indicating that α -syn transfer by cell-to-cell contact occurs in lysosomes.
427 Furthermore, this data indicates that in the case of transfer following secretion, after
428 internalization α -syn is delivered to lysosomes also in acceptor cells.

429 Overall these data indicate that hNPCs have the ability to degrade α -syn fibrils but
430 could also spread them from one cell to another, embracing the respective
431 characteristics of astrocytes and neurons (Abounit et al., 2016; Loria et al., 2017).
432 Also in hNPCs, α -syn fibrils may spread more efficiently by cell-to-cell contact as
433 previously demonstrated in neuronal cells and primary mouse neurons (Abounit et al.,
434 2016; Loria et al., 2017). Moreover, the observation of TNT-like structures with α -syn
435 puncta inside them, suggests that these connections could have a role in the
436 propagation of α -syn also in neuronal precursors.

437

438 **4.Discussion**

439 Experimental studies in rodent and primate models as well as clinical trials (Yasuhara
440 et al., 2017) have highlighted the potential of cell transplantation for treatment of
441 Parkinson's disease in order to compensate for neuronal loss and improve disease
442 symptoms (Freed et al., 2001; Wernig et al., 2008; Kriks et al., 2011; Doi et al., 2014;
443 Kikuchi et al., 2017). Several cellular populations have been considered for
444 engraftment, including human embryonic or induced pluripotent stem cell-derived
445 neuronal precursors (Xiao et al., 2016; Kikuchi et al., 2017; Zhang et al., 2018). For a
446 successful outcome, transplanted cells should display long-term survival, differentiate

447 efficiently into the appropriate neuronal phenotype and confer functional recovery.
448 However, over the years several shortcomings have raised caution, nonetheless
449 because a significant fraction of grafted cells remains in an undifferentiated precursor
450 state (Freed et al., 2001; Kikuchi et al., 2011; Kriks et al., 2011; Kikuchi et al., 2017).
451 An additional concern is the possibility that pathology may be transmitted from the
452 host brain to the graft, resulting amongst others in the emergence of pathological
453 protein species within the grafted cells (Hansen et al., 2011; Desplats et al., 2009;
454 Kordower et al., 2011; Angot et al., 2012). Therefore, careful characterization of the
455 properties of transplantable cells is of major importance.

456 Previous evidence indicates that grafted neurons can develop Lewy bodies,
457 suggesting host-to-graft disease propagation (Desplats et al., 2009; Lee et al., 2008).
458 Moreover, it is well recognized that α -syn aggregates can spread throughout the
459 nervous system in a prion-like manner (Braak et al., 2003) and different studies *in*
460 *vitro* reveal that α -syn fibrils can transfer from neuron-to-neuron (Domert et al., 2016;
461 Freundt et al., 2012), neurons-to-astrocytes and between astrocytes (Loria et al.,
462 2017). Taking into consideration this knowledge, we decided to study *in vitro*,
463 whether human iPS cell-derived NPCs may have a role in the spreading or in the
464 degradation of α -syn fibrils, as previously demonstrated for neurons and astrocytes
465 (Loria et al., 2017).

466 Our *in vitro* data demonstrates that hNPCs are able to take up α -syn fibrils very
467 efficiently, as previously shown for neurons and astrocytes. Moreover, like in neurons
468 and astrocytes, these fibrils are directed to the lysosomal compartment as soon as
469 3h after internalization (Domert et al., 2016; Freeman et al., 2013; Mak et al., 2010;
470 Sacino et al., 2017). However, in neurons α -syn fibrils that are initially directed to
471 lysosomes, may escape and accumulate within the cell through an as yet unknown

472 mechanism (Freeman et al., 2013; Victoria and Zurzolo, 2017). On the other hand, in
473 astrocytes α -syn fibrils are efficiently degraded suggesting that astrocytes play an
474 important protective role in PD and possibly other brain pathologies characterized by
475 protein aggregates (Loria et al., 2017). Given the different behavior of neurons and
476 astrocytes in response to fibril internalization, it was interesting to investigate how
477 multipotential cells, like hNPCs that can give rise to both neurons and glia -
478 astrocytes as well as oligodendrocytes – *in vitro* as well as *in vivo* (Gunhanlar et al.,
479 2018; Lim and Alvarez-Buylla, 2016; Shi et al., 2012), would perform in the presence
480 of α -syn fibrils. Our *in vitro* experiments revealed that in hNPCs, α -syn fibrils not only
481 co-localize with lysosomes but they are quite efficiently cleared from the cell within 3
482 days. Surprisingly fibril degradation does not start immediately, but initiates one day
483 after fibril loading and continues over three days. Using two specific lysosomal
484 inhibitors (E64D plus pepstatin A) we could confirm that fibril degradation occurs
485 through the lysosome pathway in hNPCs. We did not observe any difference
486 between the two genotypes (WT or A53T-syn), suggesting that the mutation at this
487 early cell state does not affect the intracellular fate of fibrils inside the lysosome nor
488 the degradation pathway. Furthermore, our co-localization analysis shows that
489 around 40% of α -syn fibrils does not co-localize with lysosomal vesicles and is not
490 inside other organelles (data not shown) suggesting that they could remain free in the
491 cytoplasm. Interestingly, this data are very similar to those reported by Flavin et al.,
492 2017 where co-localization analysis in SH-SY5Y cells shows that 55% of α -syn fibrils
493 co-localizes with chGal3, LysoTracker or both markers, while the other half co-
494 localizes with neither marker (Flavin et al., 2017).

495 The ability of misfolded α -syn to aggregate and spread throughout the brain has
496 strong implications for PD progression. Here, we demonstrate using 24h co-culture

497 experiments that hNPCs can also transfer α -syn fibrils. In particular, we observe that
498 hNPCs can form TNT-like structures. Occasionally, we also observe α -syn puncta
499 inside these structures. This suggests that the transfer of α -syn between hNPCs
500 could be TNT-mediated as previously shown in rodent neurons and human
501 astrocytes (Abounit et al., 2016; Rostami et al., 2017). However, in the absence of a
502 specific marker for TNTs, we cannot provide quantitative data to correlate the
503 number puncta inside TNTs with cell-to-cell transfer. Indeed TNTs are very dynamic
504 and fragile structures so, although we have set up specific fixation conditions
505 (Abounit et al., 2015; Sartori-Rupp et al., 2019) many of them do not resist the
506 treatment. This represents a limitation of our study and hopefully will be overcome
507 with the improvement of the techniques and the discovery of specific markers.

508 Apart from a cell contact-dependent transfer, we demonstrated that α -syn fibrils could
509 also be secreted by hNPCs, in agreement with previous studies reporting that α -syn
510 secretion could occur and could be mediated at least in part, by exosomes or other
511 extracellular vesicles (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011; Danzer
512 et al., 2012; Kunadt et al., 2015; Minakaki et al., 2018). However, in our conditions it
513 appears that the transfer mediated by secretion in the supernatant is much less
514 efficient compared with the transfer mediated by cell-to-cell contact, both in terms of
515 number of α -syn puncta transferred/cell and in terms of number of acceptor cells that
516 received α -syn. This is similar to what was previously found in mouse neuronal cells
517 and primary neurons (Abounit et al., 2016; Loria et al., 2017). Nonetheless our
518 experiments cannot rule out the possibility that local secretion could mediate part of
519 the transfer in cells that are in close apposition, in the absence of synapses. Thus
520 this hypothesis should be considered in future studies.

521 In summary, the present study provides evidence for the involvement of hNPCs in
522 both the degradation and transfer of α -syn fibrils. This opens a new perspective to be
523 considered in therapeutic strategies involving cell transplantation for treatment of PD
524 and other synucleinopathies. Our work demonstrated the ability of hNPCs to clear up
525 α -syn fibrils, suggesting that these cells could have a similar protective role as
526 observed for astrocytes (Loria et al., 2017). On the other hand, hNPCs can also
527 transfer α -syn fibrils between them and probably between hNPCs and other brain
528 cells, raising the possibility that continuous exposure to α -syn fibrils could affect the
529 lysosomal pathway and contribute to α -syn accumulation and spreading.
530 Notwithstanding that such a possible α -syn accumulation in hNPCs could influence
531 their differentiation into mature neurons. It still remains unexplored whether
532 endogenous α -syn has a role in the transition from hNPCs to mature neurons. It
533 appears that α -syn is almost entirely expressed in nerve terminals of the adult brain,
534 but it has also been found in the perikarya during development (Bayer et al., 1999;
535 Galvin et al., 2001; Raghavan et al., 2004). This may indicate that a change in the
536 subcellular localization of α -syn, could have a function in determining neuronal
537 differentiation and maturation, and particularly synapse formation. Future studies
538 should address these issues.

539

540 **Acknowledgments**

541 We are thankful to Dr. Michael Henderson for the proofread of this manuscript; all lab
542 members of CZ group for all suggestions and technical supports in this project.

543 This work was supported by the Programme Transversaux De Recherche - PTR
544 (523) from Institut Pasteur (R.M. and C.Z), Agence Nationale de la Recherche [ANR-
545 16-CE16-0019-01](C.Z), Fondation pour la Recherche Médicale [FRM-2016-

546 DEQ20160334896] (C.Z), France Alzheimer [AAP SM 2017#1674] (C.Z), LECMA-
547 VAINCRE ALZHEIMER [2016/FR-16020] (C.Z), Scientific Research on Innovative
548 Areas (Brain Protein Aging and Dementia Control) [JP26117005] from MEXT (M.H.),
549 Scientific Research on Brain Mapping by Integrated Neurotechnologies for Disease
550 Studies (Brain/MINDS) [JP14533254] from AMED (M.H.), JST CREST (JP18071300)
551 (M.H.),Brain Science Foundation (T.N.).

552

553 **Declaration of interests**

554 The authors declare no competing interests.

555

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741

742 **Figure legends**

743

744 **Fig. 1 Time course of α -syn fibrils internalization in hNPCs.**

745 **A** Immunostained for fluorescent Alexa 568- α -syn fibrils (red) and *Wheat germ*
746 *agglutinin* (WGA) (gray) in WT (upper) and A53T (bottom) hNPCs at different time
747 points. Scale bar 10 μ m.

748 **B.** Quantification of the number of Alexa 568-positive cells during the time. Data are
749 shown as mean \pm s.e.m from three independent experiments. Detection was
750 determined by confocal microscopy and ICY software.

751 **C** Quantification of the average number and size per cells of α -syn fibrils puncta in
752 hNPCs (WT left bar graph, A53T right bar graph) at different time points. Detection
753 was determined by confocal microscopy and ICY software. Ns, not significant by one-
754 way Anova, Tukey's multiple comparison test. Data are shown as mean \pm s.e.m from
755 three independent experiments.

756

757 **Fig. 2 Cytotoxicity assay in hNPCs.**

758 Cell toxicity was measured by LDH release in WT (left bar graph) and A53T (right bar
759 graph) hNPCs. The bar graph represents the percentage of cytotoxicity in control and
760 α -syn fibrils (0.3 μ M) condition at 16h, 1 day and 3 days. There were no significant
761 differences between control (black bar) and α -syn -loaded (gray bar) cells at any of
762 the time points evaluated. Ns, not significant by one-way Anova, Tukey's multiple
763 comparison test. Data are shown as mean \pm s.e.m from three independent
764 experiments.

765

766 **Fig.3 α -syn fibrils are found in lysosomal vesicles in the cytosol of hNPCs at 3,**
767 **6, 12 and 16h following fibril loading.**

768 **A** Percentage of co-localization between LAMTOR4 (marker for lysosome) and Alexa
769 568 α -syn fibrils considering Pearson correlation coefficient at different time points
770 (WT left bar graph, A53T right bar graph). Data are shown as mean \pm s.e.m from
771 three independent experiments.

772 **B** Representative confocal images of co-localization in WT (upper panel) and in A53T
773 hNPCs (bottom panel). LAMTOR4 green, α -syn fibrils red, WGA gray. Intracellular
774 localization of α -syn puncta in hNPCS was confirmed with the magnified orthogonal
775 cross-section view (x/y , x/z , and y/z axes) that corresponds to a single slice of the Z-
776 stack. Scale bars represent 10 μ m.

777

778 **Fig.4 α -Syn fibrils degradation in hNPCs.**

779 **A** Representatives confocal images showing the degradation of fibrils at 16h, 1 day
780 and 3 days in WT (upper panel) and A53T hNPCs (bottom panel); α -syn fibrils red,
781 WGA gray. Scale bar 10 μ m.

782 **B** Representative immunoblot of α -syn fibril over time in WT and A53T hNPCs. Cell
783 lysates (10 μ g) from control and α -syn fibril-loaded hNPCs overnight were collected at
784 different time points (16h, 1 day and 3 days) and immunoblotted against α -syn (Syn-
785 1) and α -tubulin (as a loading control). Bar graphs show the total amount α -syn fibrils
786 over time in WT (left panel) and A53T (right panel) hNPCs. Data represent the
787 mean \pm sem of at least three independent experiments (One-way Anova, Tukey's
788 multiple comparison test, * $p < 0.05$, ** $p < 0.01$)

789 **C** Representative immunoblot of α -syn fibril without (control) or in the presence of
790 E64D (20 μ M) plus pepstatin A (20 μ M) (E+P) for 36h in WT and A53T hNPCs.
791 Immunoblotted against α -syn (Syn-1) and GAPDH (as a loading control). Data
792 represent the mean \pm sem of two independent experiments (One-way Anova,
793 Tukey's multiple comparison test, * $p < 0.05$, ns = not significant).

794

795 **Fig.5 α -syn fibrils transfer efficiently between hNPCs after 24h in co-culture**
796 **experiments.**

797 **A** Schematic of the co-culture system design between WT or A53T hNPC (donor
798 cells) and LV-GFP WT hNPC (acceptor cells).

799 **B** Representative Z-stack projection of confocal images of 24h co-cultured hNPCs.
800 Intracellular localization of α -syn puncta in hNPCS was confirmed with the magnified
801 orthogonal cross-section view (x/y , x/z , and y/z axes) that corresponds to a single
802 slice of the Z-stack. Scale bars represent 10 μ m. D= donor cells, A= acceptor cells.
803 The yellow arrows show some examples of α -syn puncta inside acceptor cells.

804 **C** Bar graph showing the number α -syn puncta per acceptor hNPCs after 24 h in co-
805 culture experiment. Ns, not significant by Mann Whitney test. Data show mean \pm sem
806 of three independent experiments.

807 **D** Schematic of the secretion experiment design between WT or A53T hNPC (donor
808 cells) and LV-GFP WT hNPC (acceptor cells).

809 **E** Representative Z-stack projection of confocal images of acceptor hNPCs after 24h
810 of conditioned medium. The yellow arrows show some examples of α -syn puncta.

811 **F** Bar graph showing the number α -syn puncta per acceptor hNPCs after 24 h
812 secretion experiment. Ns, not significant by Mann Whitney test. Data show
813 mean \pm sem of three independent experiments.

814 **G** Bar graph showing the percentage of acceptor cells positive for α -syn puncta in co-
815 culture and secretion system for both genotypes. Ns, not significant, *** $p < 0.001$ by
816 Mann Whitney test. Data show mean \pm sem of three independent experiments.

817 **H** Representative confocal images showing TNT-like structures (yellow arrows)
818 between hNPCs and in particular α -syn puncta inside of TNTs (magnification).

819

820 **Fig.S1 Characterization of hNPCs.**

821 **A** Immunostaining for Nestin, SOX2, Pax6 (markers of neural progenitor cells), DCX
822 (marker of early born neurons), Lmx1a and FOX2a (markers of dopaminergic lineage
823 specification). Scale bar 40 and 10 μ m.

824 **B** Detection of SNCA gene by PCR and the mutation A53T/G209A from human iPCS
825 cell-derived dopaminergic NPCs.

826 **C** Neuronal differentiation of hNPCs at 30 days. Immunostaining for TH (Tyrosine
827 hydroxylase) and MAP2 (dendritic marker). Nuclei are visualized with DAPI. Scale
828 bar 10 μ m.

829

830 **Fig.S2 Immunofluorescence of samples without (control) or with the treatment**
831 **with lysosomal inhibitors E64D plus pepstatin A (E+P).**

832 Lyotracker Deep Red staining and Alexa- 568 α -syn fibril after internalization (16H),
833 and after 36 hours in control and in the presence of the inhibitors (E+P).

834 Scale bars represent 10 μ m.

835

836 **Fig. S3 Internalization control of α -syn puncta in hNPCs.**

837 Representative Z-stack projection of confocal images of hNPCs treated with 0.3 μ M
838 of Alexa- 568 α -syn sonicated fibrils overnight, washed with trypsin (trypsin diluted
839 1:3 PBS) and re-plated. Intracellular localization of α -syn puncta in hNPCs was
840 confirmed with the magnified orthogonal cross-section view (x/y , x/z , and y/z axes)
841 that corresponds to a single slice of the Z-stack shows clearly the absence of fibrils
842 still attached on the plasma membrane. Scale bars represent 10 μ m.

843

844 **Fig. S4 Alexa 568 α -syn fibrils confirmed using α -syn Ab in co-culture**
845 **condition.**

846 Representative Z-stack projection of confocal images. The arrows show some
847 examples of co-localization between Alexa 568 α -syn fibrils (red), α -syn revealed by
848 Ab (far red) in acceptor cells after 24h of co-culture. Scale bar 10 μ m.

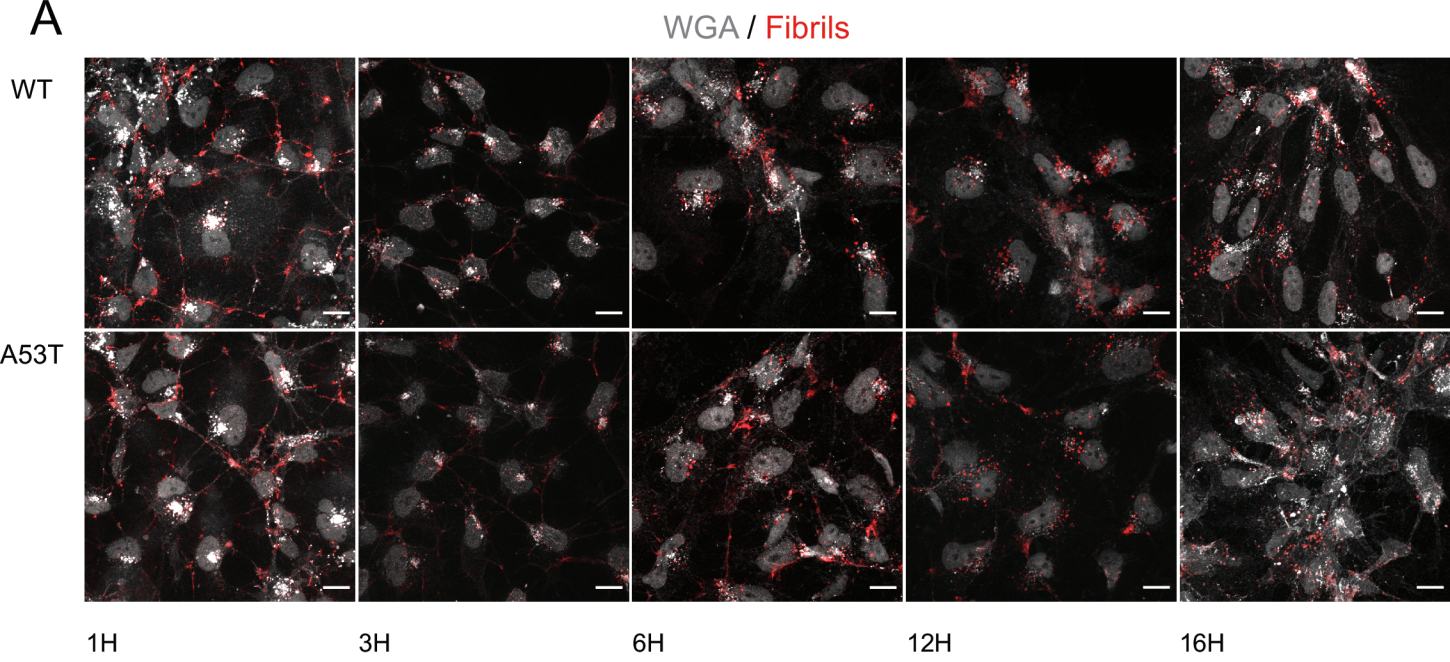
849

850 **Fig.S5 Localization of Alexa 568 α -syn fibrils into lysosomes in acceptor cells**
851 **in co-culture and secretion condition.**

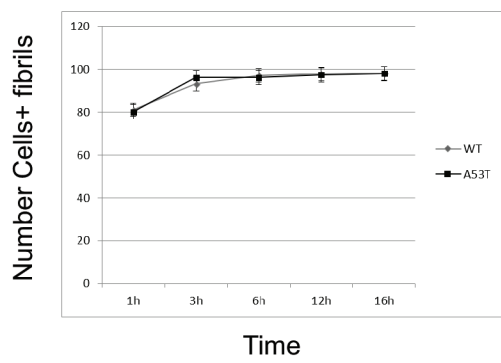
852 Representative Z-stack projection of confocal images. Immunostaining for lysosomes
853 using Lyotracker Deep Red in acceptor cells and donor cells in co-culture (upper
854 panel) and secretion (bottom panel) condition. The arrows show some examples of
855 co-localization between fibrils and lysosomes. Scale bar 10 μ m. Percentage of co-

856 localization between LysoTracker Deep Red and Alexa 568 α -syn fibrils into acceptor
857 cells considering Pearson correlation coefficient.

858



B



C

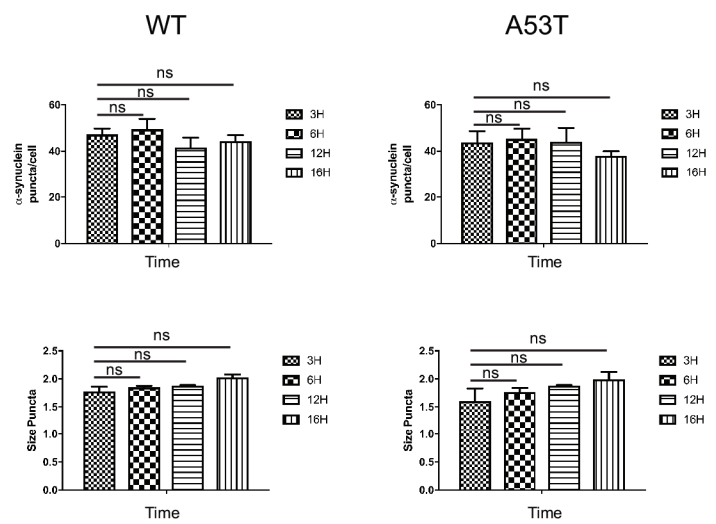


Figure 1

A

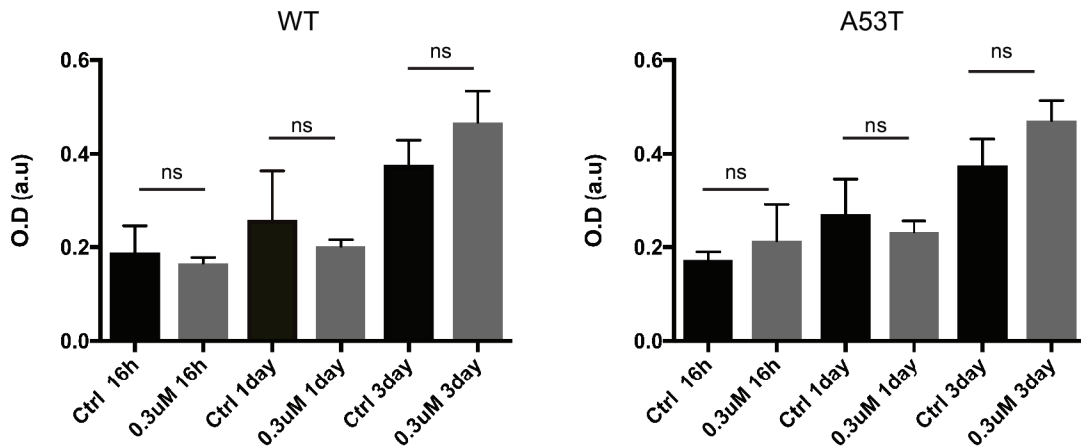
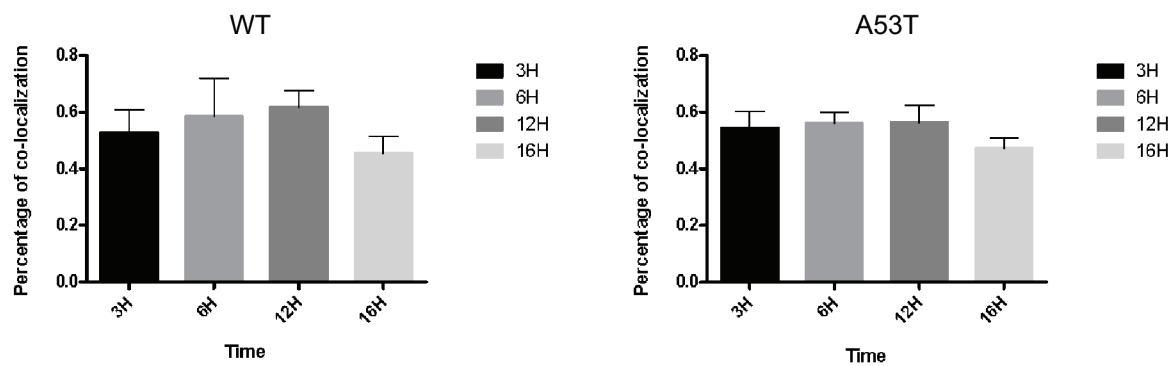


Figure 2

A



B

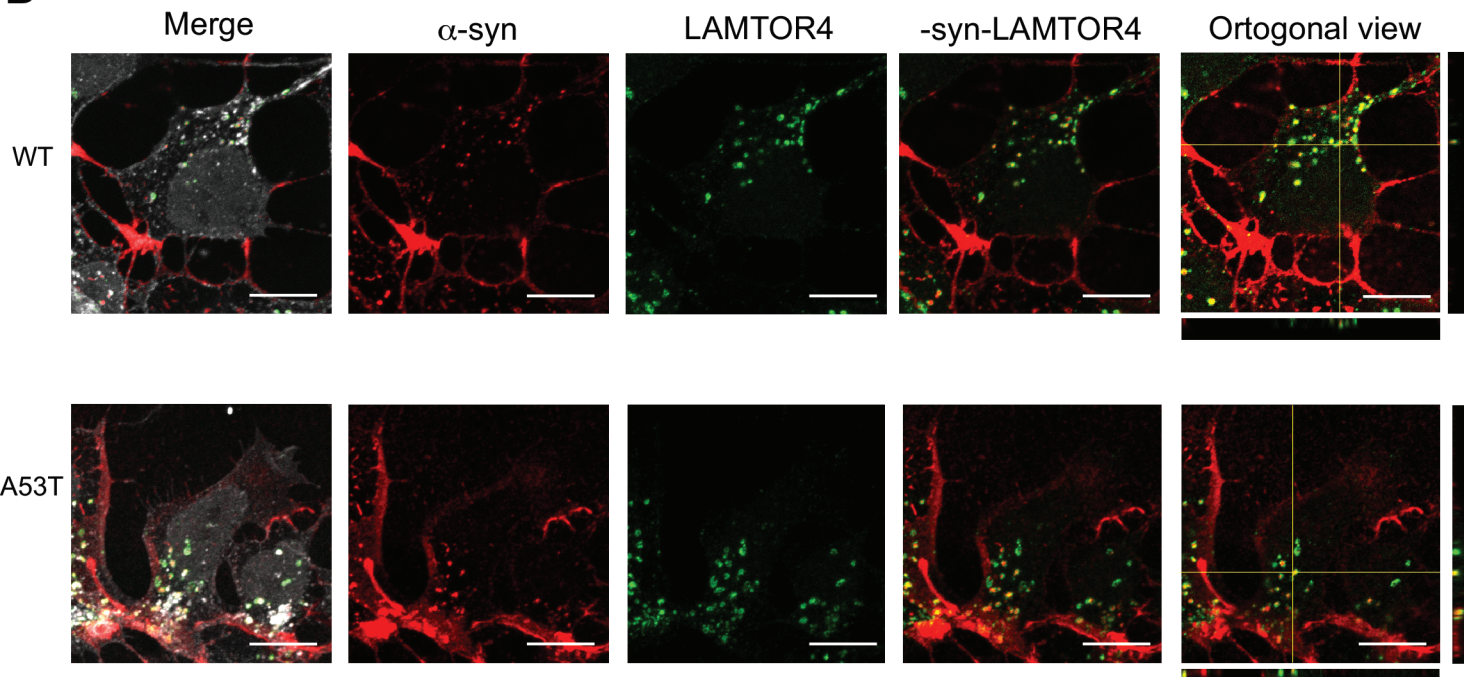
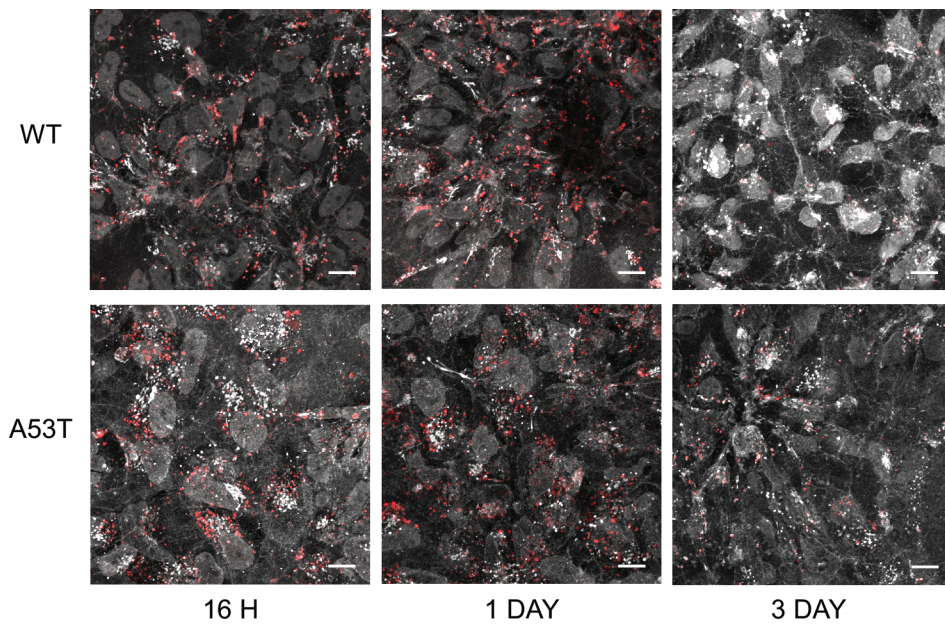


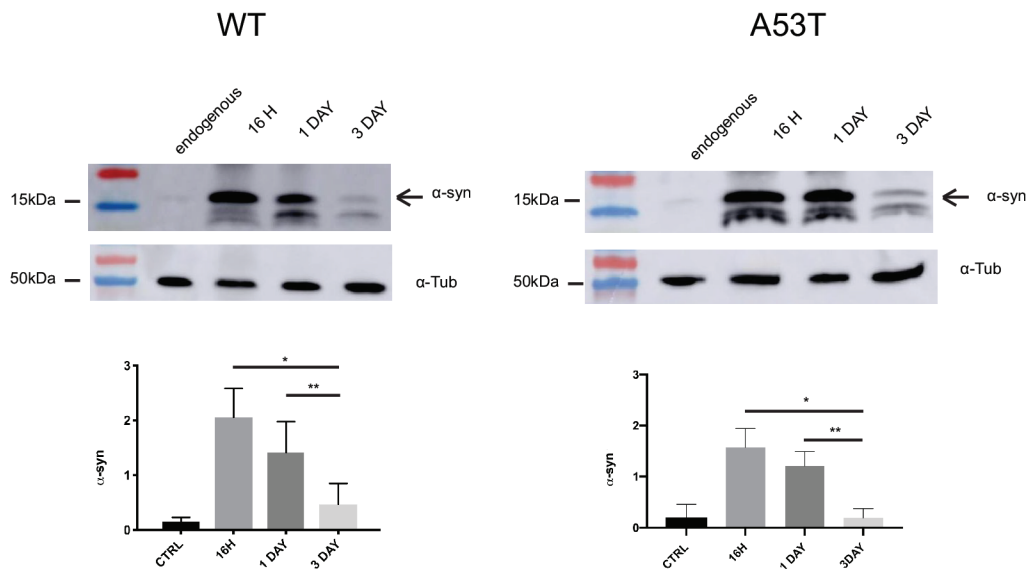
Figure 3

A

Fibrils / WGA



B



C

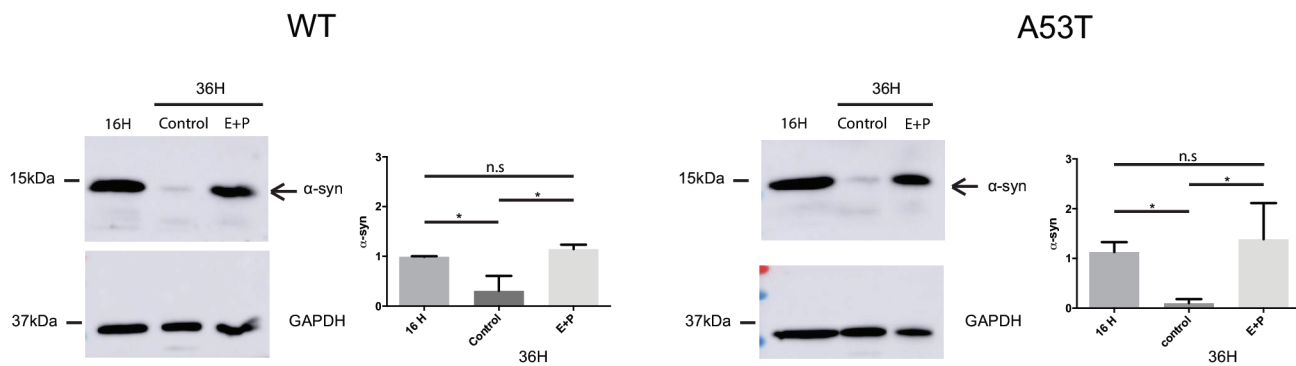


Figure 4

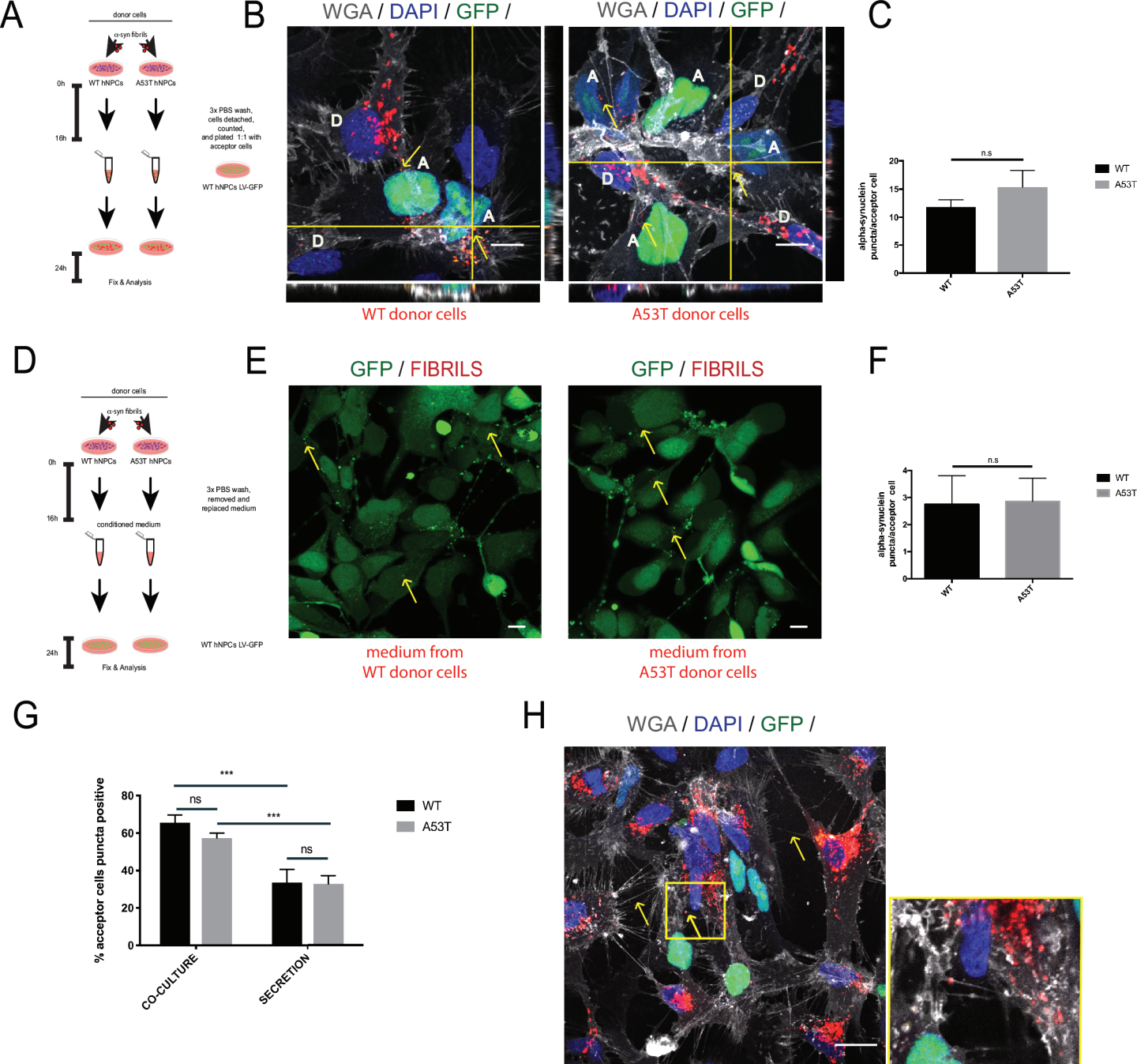


Figure 5