

1 Alpha-synuclein phosphorylation at serine 129 occurs after initial protein deposition
2 and inhibits seeded fibril formation and toxicity

3 Simona S. Ghanem^{*,1}, Nour K. Majbour^{*,1}, Nishant N. Vaikath^{*,1}, Mustafa T.
4 Ardah², Daniel Erskine³, Nanna Møller Jensen^{4,5}, Muneera Fayyad¹, Indulekha P.
5 Sudhakaran¹, Eftychia Vasili⁶, Katerina Melachroinou⁷, Ilham Y. Abdi¹, Ilaria
6 Poggiolini¹, Patricia Santos⁶, Anton Dorn⁸, Paolo Carloni^{8,9}, Kostas
7 Vekrellis⁷, Johannes Attems³, Ian McKeith³, Tiago F. Outeiro^{6,10,11}, Poul Henning
8 Jensen^{4,5}, Omar M. A. El-Agnaf^{*,1}

9

10 ¹Neurological Disorders Research Center, Qatar Biomedical Research Institute
11 (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation, Doha, Qatar

12 ²Department of Biochemistry, College of Medicine and Health Science, United Arab
13 Emirates University, Al Ain, United Arab Emirates

14

15 ³Translational and Clinical Research Institute, Newcastle University, Newcastle upon
16 Tyne, UK

17 ⁴Danish Research Institute of Translational Neuroscience – DANDRITE, Aarhus
18 University, Aarhus, Denmark

19

20 ⁵Department of Biomedicine, Aarhus University, Aarhus, Denmark

21

22 ⁶Department of Experimental Neurodegeneration, Center for Biostructural Imaging of
23 Neurodegeneration, University Medical Center Göttingen, Göttingen, Germany

24

25 ⁷Center of Basic Research, Biomedical Research Foundation of the Academy of
26 Athens, Athens, Greece

27

28 ⁸Department of Physics, RWTH Aachen University, Aachen, Germany

29

30 ⁹Computational Biomedicine, Institute of Advanced Simulation IAS-5 and Institute of
31 Neuroscience and Medicine INM-9, Germany; JARA Institute: Molecular
32 Neuroscience and Imaging, Institute of Neuroscience and Medicine INM-11,
33 Forschungszentrum Jülich GmbH, 52425 Jülich, Germany.

34

35 ¹⁰Max Planck Institute for Experimental Medicine, Göttingen, Germany

36

37 ¹¹Translational and Clinical Research Institute, Newcastle University, Newcastle
38 upon Tyne, United Kingdom

39

40 ^{*} Simona S. Ghanem, Nour K. Majbour and Nishant N. Vaikath contributed equally to
41 this article.

42 ^{*} Correspondence: Omar El-Agnaf, Neurological Disorders Research Center, Qatar
43 Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU),
44 Education City, Qatar Foundation, P.O. Box 5825 Doha, Qatar, Tel: (+974) 44545664,
45 E-mail: oelagnaf@hbku.edu.qa

46 **SUPPLEMENTARY METHODS**

47 *Expression and purification of recombinant human α -syn*

48 Full-length recombinant human α -syn was expressed in Escherichia coli BL21 (DE3)
49 using the bacterial expression vector pRK172. Following expression and
50 sedimentation, the bacterial pellets from 1 liter of Terrific broth (TB) were homogenized
51 and sonicated in 50 ml of high-salt buffer (0.75 M NaCl, 10 mM Tris, pH 7.6, 1 mM
52 EDTA) containing a cocktail of protease inhibitors (Thermo Scientific), heated to 100°C
53 for 10 min, and centrifuged at 5300g for 20 min. The solution was dialysed overnight
54 against the buffer used for gel filtration chromatography (50 mM NaCl, 10 mM Tris, pH
55 7.6, 1 mM EDTA), following which, the volume was reduced to 5 ml using a Pierce
56 protein concentrator (10K MWCO; ThermoFisher Scientific) according to the
57 manufacturer's instructions. All proteins were purified by size exclusion using a
58 Superdex 200 gel filtration column (GE Healthcare). The clean fractions were pooled,
59 exchanged with a buffer (10 mM Tris pH 7.6, 25 mM NaCl, 1 mM EDTA, 1 mM PMSF)
60 for ion exchange chromatography by dialysis overnight, and were applied onto a
61 HiTrap Q column (GE Healthcare) and eluted in 10 mM Tris pH 7.6 using a linear
62 gradient of 0.025–1.0 M NaCl. For preparation of α -syn monomers, the protein went
63 through 100 KDa filters to remove any high molecular weight proteins. Purified
64 fractions were pooled, and protein concentrations were determined using the Pierce
65 BCA protein assay kit (ThermoFisher Scientific).

66 *Aggregation of α -syn In Vitro*

67 The purity of α -syn was >95% as estimated by SDS gels. α -Syn samples were placed
68 in 1.5-mL sterile polypropylene tubes and sealed with parafilm, followed by incubation
69 at 37 °C for several days with continuous shaking at 800 rpm in a Thermomixer
70 (Eppendorf). The samples were collected at the indicated time points, while the

71 aggregation of α -syn was monitored by Th-S binding assay. The samples were stored
72 at $-80\text{ }^{\circ}\text{C}$ for future analyses. Recombinant monomeric α -syn was mixed with various
73 percentages of in vitro prepared monomeric pS129- α -syn (100%, 50%, 20%, 5%, or
74 0%) in 1.5-mL sterile polypropylene tubes, followed by incubation for up to 20 d.

75 *Th-S Assay*

76 Th-S binding assay was used to study α -syn fibril formation. Being a fluorescent dye,
77 Th-S interacts with fibrils containing β -sheet structures. The sample (10 μL) was
78 diluted in 40 μL of Th-S (20 μM) in PBS and the mixture was dispensed in a 384-well,
79 untreated black microplate (Nunc). Fluorescence was measured in a microplate reader
80 (Perkin-Elmer Envision) with the excitation and emission wavelengths at 450 and 510
81 nm, respectively.

82 *Preparation of α -syn Pure Fibrils and Pure PFFs*

83 Monomeric α -syn (100 μM) was aggregated as described above for 7 d. For
84 preparation of pure fibrils, crude α -syn fibril sample was spun at $10,000 \times g$ for 10 min
85 at $4\text{ }^{\circ}\text{C}$ in a refrigerated microfuge (Eppendorf). The supernatant was then discarded,
86 and the pellet was washed twice and finally resuspended in $1\times$ PBS. For preparation
87 of pure PFFs, the pure fibrils were fragmented on ice by ultrasonication using a Sonic
88 Ruptor 250, equipped with a fine tip (2-s pulses, output of 40 W for 5 min). For
89 measurement of α -syn concentration of fibrils and PFFs, the samples were denatured
90 with equal volume of 6 M guanidine-HCl and quantified using Pierce BCA protein
91 assay kit (ThermoFisher Scientific).

92 *In Vitro Phosphorylation of α -syn PFFs (pS129- α -syn PFFs)*

93 The fibrils produced by incubating α -syn for 7 d at $37\text{ }^{\circ}\text{C}$ with continuous shaking,
94 were centrifuged at 14,000 rpm for 10 min to remove monomers and soluble
95 oligomers. The pellets were then washed three times with ultrapure water to remove

96 any salt traces and resuspended with ultrapure water, then subjected to brief
97 sonication. The protein estimation was performed by dissolving the sonicated pure
98 PFFs in 6 M guanidine-HCL and then by BCA assay. The PFFs were then
99 phosphorylated as follows: 100 μ M of PFFs was phosphorylated at serine 129 (S129)
100 using PLK2 (Invitrogen) in 1 \times phosphorylation buffer (20 mM Hepes, 1.09 mM ATP, 2
101 mM DTT, 10 mM MgCl₂, and 1 μ g PLK2) and incubated at 37 °C for 48 h with gentle
102 shaking (reaction final volume is 100 μ L). The sample was then sonicated briefly and
103 stored at -80 °C.

104 *Isolation of Soluble Fraction from Brain Tissues*

105 Brain tissues derived from the temporal and frontal cortex were homogenized on ice
106 with a glass tissue homogenizer at 10% (wt/vol) in TBS (20 mM Tris-HCl pH 7.4, 150
107 mM NaCl) and 5 mM EDTA with protease and phosphatase inhibitors (ThermoFisher
108 Scientific). Samples were centrifuged at 3,000 \times g at 4 °C for 30 min. The collected
109 supernatant represents the TBS-soluble fraction. The total protein concentration was
110 measured by BCA assay (ThermoFisher Scientific). Aliquots of 0.1 mg/mL were
111 prepared and stored at -80 °C.

112 *Tissue culture of WT BE(2)-M17 human neuroblastoma cells*

113 Human neuroblastoma cells (WT BE(2)-M17) were cultured in Dulbecco's
114 MEM/Nutrient Mix F-12 (1:1) (Hyclone) containing 10% FBS (Hyclone) and 1%
115 penicillin-streptomycin (P/S; 10,000 U/ml penicillin, 10 mg/ml streptomycin-Sigma).
116 The cells were maintained at 37°C in a humidified incubator with 5% CO₂/95% air.

117 *Tissue culture of WT SH-SY5Y human dopaminergic neuroblastoma cells*

118 Human dopaminergic neuroblastoma cells (WT SH-SY5Y) were cultured in
119 Dulbecco's MEM/Nutrient Mix F-12 (1:1) (Hyclone) containing 15% FBS (Hyclone),
120 1% penicillin-streptomycin (P/S; 10,000 U/ml penicillin, 10 mg/ml streptomycin-

121 Sigma), and supplemented with 1% non-essential MEM amino acid supplement
122 (Gibco) and 2 mM freshly prepared glutamine. The cells were maintained at 37°C in a
123 humidified incubator with 5% CO₂/95% air.

124 *Measurement of Cell Viability*

125 To assess the cytotoxic effect of different α -syn species, cells were plated at a density
126 of 15,000 cells (100 μ L per well) in a 96-well plate. After 24 h, the medium was
127 replaced with 100 μ L of MEM-RS (HyClone) serum-free medium containing different
128 solutions of α -syn species and treated for 48 h. A total of 20 μ L of 3-(4,5-
129 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) (6
130 mg/mL) was dispensed into each well, and incubated for 4.5 h. This was replaced with
131 100 μ L per well of lysis buffer (15% SDS, 50% N, *N*-dimethylformamide, pH 4.7)
132 overnight. The absorbance values at 590 nm were measured in a microplate reader
133 (Perkin-Elmer). For experiments assessing the toxic effect of seeded polymerization,
134 cells were treated in serum-free medium containing different solutions of α -syn species
135 (pure fibrils and pure PFFs) for 1 h and monomeric α -syn was then added, followed
136 by 48-h incubation.

137 *Development and validation of specific antibody against non-phosphorylated S129- α -* 138 *syn (WT- α -syn) aggregates*

139 Using hybridoma technology for monoclonal antibody development (1), a mouse
140 monoclonal antibody that specifically recognizes WT- α -syn when it is not pS129 was
141 generated (4B1). A short synthetic peptide, designed over the region of interest (a.a.
142 125–133; CYEMPSEEGY), was used as the antigen. This peptide was solubilized in
143 PBS and conjugated to KLH carrier protein. Experimental procedures using mice were
144 carried out in accordance with Laboratory Animal Research Center (LARC), Qatar
145 University (QU), Qatar, according to the QU institutional ethical rules and regulations

146 and approved by QU—IACUC & IBC. Following hybridoma technology, the
147 monoclonal antibody (mAb 4B1) was generated. Using protein-G agarose affinity
148 chromatography (Sigma-Aldrich), antibody was purified from culture supernatant.
149 Among several single cell cloning steps and characterization, 4B1 antibody was
150 selected for this study. The purity of 4B1 antibody was assessed using SDS-PAGE
151 under reducing conditions.

152 *Generation of 4-oxo-2-nonenal (ONE)-, 4-hydroxy-2-nonenal (HNE)- α -syn oligomers*
153 HNE-/ONE- α -syn oligomers were prepared as previously described (2). For
154 generation of HNE-/ONE- α -syn oligomers, α -syn was dialysed against 50 mM
155 disodium hydrogen phosphate, pH 8.5 followed by filtration using 100-kDa MWCO
156 micron spin filter (Millipore) to get rid of high molecular weight aggregates. HNE or
157 ONE (Abcam) was then added to α -syn monomers (140 μ M) to get a final molar ratio
158 of 30:1 (HNE/ONE: α -syn) followed by incubation of the samples at 37°C for 18 hours
159 without shaking. The samples were then centrifuged at 16,900 x g for 5 min to get rid
160 of any high molecular aggregated species. The supernatant containing the oligomeric
161 species was then purified by size exclusion chromatography on a Superdex 200 gel
162 filtration column (GE healthcare) equilibrated with 20 mM Tris pH 7.4, 0.15 M NaCl
163 buffer. The eluted peaks fractions corresponding to the oligomeric fraction were pooled
164 and quantified using BCA protein assay kit after solubilizing the oligomers in equal
165 volume on 6 M GnHCl.

166 *Filter retardation assay*

167 Filter retardation assay was performed using a Minifold 48 slots (GE Healthcare Life
168 Sciences). Each protein (50 μ l) at a final concentration of 1 μ g/ml was loaded into each
169 slot on a nitrocellulose membrane that has been pre-soaked in PBS. Samples were
170 allowed to absorb onto the nitrocellulose membrane and then slots were washed with

171 1 ml of PBS. Membranes were then probed with relevant antibodies, and developed
172 with SuperSignal West Pico Chemiluminescent Substrate Kit.

173 *Inhibition ELISA*

174 A 384-well black MaxiSorb microplate (Nunc) was coated with 1 ug/ml of α -syn
175 monomers in 0.2 M NaHCO₃ pH 9.6 with overnight incubation at 4°C. 4B1 antibody at
176 50ng/ml was pre-incubated with serial dilutions of α -syn monomers or aggregates with
177 continuous rolling for 2 hours. The antibody-protein mixture was then loaded to the
178 antigen-coated plate and incubated for 10 min at RT. After washing step, goat anti-
179 mouse IgG-HRP (1:20,000, Jackson) was added for 1 hour to be later detected
180 using SuperSignal ELISA Femto Chemiluminescent Substrate Kit.

181 *Sandwich ELISA*

182 A 384-well ELISA microplate was coated with 4B1 antibody at 0.5 μ g/ml overnight at
183 4°C in 0.2 M NaHCO₃ pH 9.6. After incubating the plate with 100 μ l/well of blocking
184 buffer for 2 hours at 37°C, serial dilutions of α -syn monomers, pS129- α -syn
185 monomers, or different α -syn oligomers were added to corresponding wells, and
186 incubated overnight at 4°C. Biotinylated 3G7 (mouse mAb for total α -syn) (3) was
187 added as detection antibody and incubated at 37°C for 2 h followed by a washing step
188 and incubation for 1 hour at 37°C with streptavidin-HRP (Sigma). The plate was then
189 washed and 50 μ l/well of an enhanced chemiluminescent substrate (SuperSignal
190 ELISA Femto, Pierce Biotechnology) was added to corresponding wells. The
191 chemiluminescence, expressed in relative light units, was immediately measured
192 using Envision plate reader (PerkinElmer Envision).

193 *Western blot analysis on SNCA transgenic and SNCA null mouse brain tissue*

194 SNCA transgenic and SNCA null mouse brain tissues (10 μ g) were analyzed on 12%
195 SDS-PAGE and immunoprobng with appropriate antibodies. These include

196 monoclonal antibodies against mouse α -syn Syn1 (BD Biosciences) and non-pS129-
197 α -syn (4B1) in addition to antibody C4 against β -Actin (Sc-47778, Santa Cruz
198 Biotechnology) as a protein loading control.

199 *Immunoprecipitation*

200 TBS fractions of three DLB brain lysates (100 μ g of total protein/sample) were
201 incubated overnight at 4°C with 100 μ g/ml of 4B1 antibody under gentle rotation. This
202 was followed by a 2-hour incubation with 100 μ l Pierce Protein G agarose (Thermo
203 Scientific) at room temperature under gentle rotating conditions. Samples were then
204 centrifuged for 3 min at 2500 x g and the supernatant was collected, then the beads
205 were washed three times with PBS.

206

207 **SUPPLEMENTARY RESULTS**

208 Given that the effect was independent of pure PFFs/fibrils concentration, cell viability
209 was evaluated upon varying both the concentration of PFFs and fibrils (lower
210 concentration scale, of 0.0001-10 μ M) in the presence of a constant concentration of
211 α -syn monomers and *vice versa* (Supplementary Fig. 4). As shown in Supplementary
212 Fig. 4A, B, pure PFFs and fibrils decrease cell viability in a concentration-dependent
213 fashion, with a more pronounced toxic effect in the presence of monomeric α -syn (final
214 concentration of 10 μ M), with PFFs being more toxic than fibrils. Additionally, different
215 concentrations of monomeric α -syn (1-20 μ M) on cell viability were assessed, with
216 cells first treated with a constant concentration (2 μ M) of either pure fibrils or PFFs. As
217 indicated in Supplementary Fig. 4C, D, the addition of monomers seems necessary
218 for cell toxicity when pre-treated with pure fibrils or PFFs since α -syn monomers alone
219 had no effect (4).

220 To further emphasize the role of monomeric α -syn in inducing the nucleation
221 polymerization process of α -syn, the effect of pure PFFs and fibrils on the viability of
222 BE(2)-M17 WT cells with siRNA silenced endogenous α -syn was also examined.
223 Immunoblotting and its corresponding quantification of α -syn expression are shown in
224 Supplementary Fig. 5A, B. The results demonstrated that the cells whose endogenous
225 α -syn was knocked down were less susceptible to the toxic effects of pure fibrils
226 (Supplementary Fig. 5C) and PFFs (Supplementary Fig. 5D). However, the treatment
227 of cells with monomeric α -syn at all given concentrations had similar effects in siRNA-
228 transfected and control cells (Supplementary Fig. 5E). The data confirm the important
229 role of monomeric α -syn in aggregation-induced toxicity.

230 *Effect of immunodepleting WT- α -syn by 4B1 in DLB brain lysates*

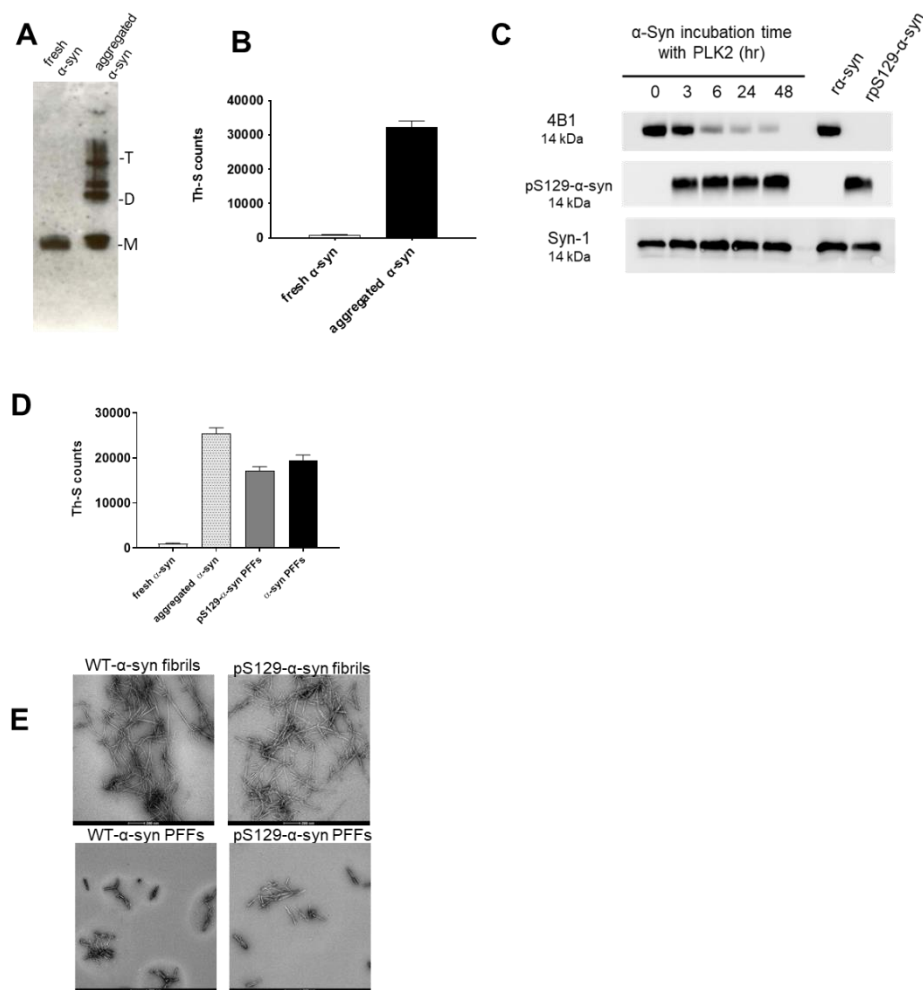
231 Western blot analysis on TBS fractions of PD/DLB lysates (Supplementary Figure 3A)
232 showed no detection of pS129- α -syn. Thus, immunodepleting WT- α -syn using 4B1
233 from brain lysates of three DLB cases was performed. The native gel analysis
234 (Supplementary Figure 3B) showed that brain lysates contained high molecular
235 weights of WT- α -syn species, but immunodepleted samples did not. Interestingly, 4B1
236 did not detect monomeric WT- α -syn in DLB brain lysates or recombinant WT- α -syn,
237 confirming its specificity for aggregated WT- α -syn. In contrast, Syn-140 (sheep
238 polyclonal antibody) (5) detected equal amounts of different α -syn species in both
239 immunodepleted and untreated DLB samples. A further validation of the
240 immunodepleted samples was performed using oligomeric ELISA (Supplementary
241 Figure 3C), with low signals of WT-syn oligomers compared to untreated
242 samples. Additionally, both brain lysates and their immunodepleted corresponding
243 samples were tested using RT-QuIC assay, showing no evidence of seeded
244 aggregation in the immunodepleted groups, as illustrated in Supplementary Figure 3D.

Case ID	Sex	Age at onset	Age at death	McKeith	Braak LB	Thal phase	Braak NFT
Case 1	F	77	81	Neocortical	6	3	4
Case 2	F	67	73	Neocortical	6	4	3
Case 3	M	64	83	Neocortical	6	5	4
Case 4	M	64	86	Neocortical	5	4	3
Case 5	M	72	78	Neocortical	6	4	3
Case 6	M	78	86	Neocortical	5	3	1
Case 7	M	62	73	Neocortical	6	1	3
Case 8	M	84	90	Neocortical	6	5	3
Case 9	F	85	87	Neocortical	6	5	2
Case 10	M	65	82	Neocortical	6	3	2
Case 11	M	64	74	Neocortical	5	5	2
Case 12	M	67	78	Neocortical	6	1	3
Case 13	M	65	73	Neocortical	6	5	3
Case 14	M	70	79	Neocortical	6	4	2
Case 15	F	92	92	Neocortical	5	5	2
Case 16	M	61	75	Neocortical	5	0	1
Case 17	M	52	74	Neocortical	6	5	3
Case 18	M	80	82	Neocortical	6	1	1
Case 19	M	69	77	Neocortical	6	2	3
Case 20	M	66	78	Neocortical	6	2	2
Case 21	M	83	92	Neocortical	6	2	3
Case 22	M	85	87	Limbic	5	5	4
Case 23	M	51	68	Neocortical	6	4	2
Case 24	M	77	81	Neocortical	6	4	3
Case 25	M	81	89	Neocortical	6	3	2
Case 26	F	85	93	Neocortical	6	5	4
Case 27	M	77	84	Neocortical	6	2	3
Case 28	F	91	98	Limbic	5	2	2
Case 29	F	52	76	Neocortical	6	3	2
Case 30	F	52	82	Neocortical	6	3	3

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247 **Supplementary Table 1.** Demographics of the neuropathology cohort. "McKeith"
248 refers to Newcastle-McKeith Lewy body stage (McKeith et al, 2017, PMID: 28592453),
249 "Braak LB" refers to Lewy body stage (Braak et al, 2003; PMID: 12498954), "Thal
250 phase" to amyloid-beta phase (Thal et al, 2002; PMID: 12084879) and "Braak NFT" to
251 neurofibrillary tangle pathology stage (Braak et al, 2006; PMID: 16906426).

Supplementary Fig. 1



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253 **Supplementary Figure 1. Characterization of *in vitro* prepared pS129- α -syn. A.**
 254 **Characterization of monomeric and aggregated α -syn by immunoblotting. B. Th-S**
 255 **fluorescence readings of the monomeric and aggregated α -syn samples. C.**
 256 **Immunoblotting for α -syn samples incubated with PLK2 at different time points (0, 3,**
 257 **6, 24, and 48 hr). Phosphorylation at S129 was detected by Abcam ab51253 pS129-**
 258 **α -syn-specific antibody. WT- α -syn was detected by 4B1 and total α -syn by Syn-1. D.**
 259 **Fibril content estimation in the pS129- α -syn PFFs, WT- α -syn PFFs and aggregated α -**
 260 **syn by Th-S fluorescence. The assay was performed in triplicates and, the means \pm**
 261 **standard deviations are shown. E. Electron microscopy images of negatively stained**
 262 **samples of WT- and pS129- α -syn fibrils and PFFs. Scale bar 200 nm.**

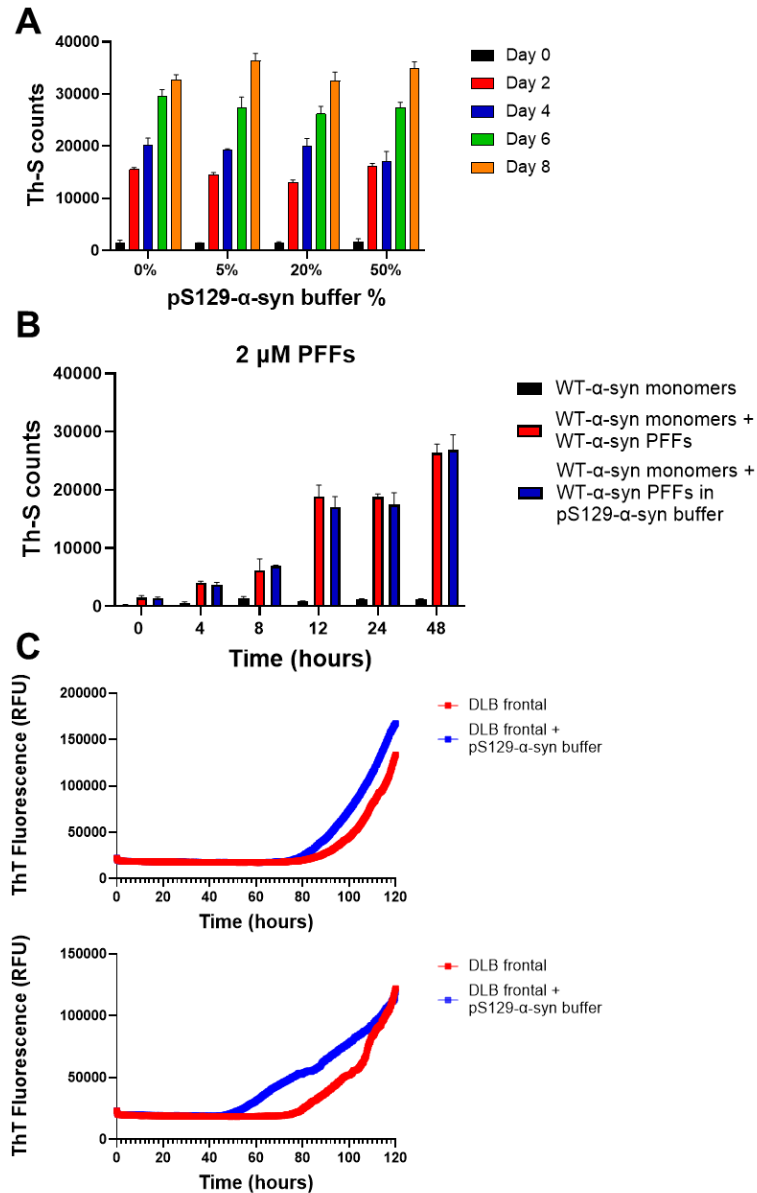
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Supplementary Fig. 2



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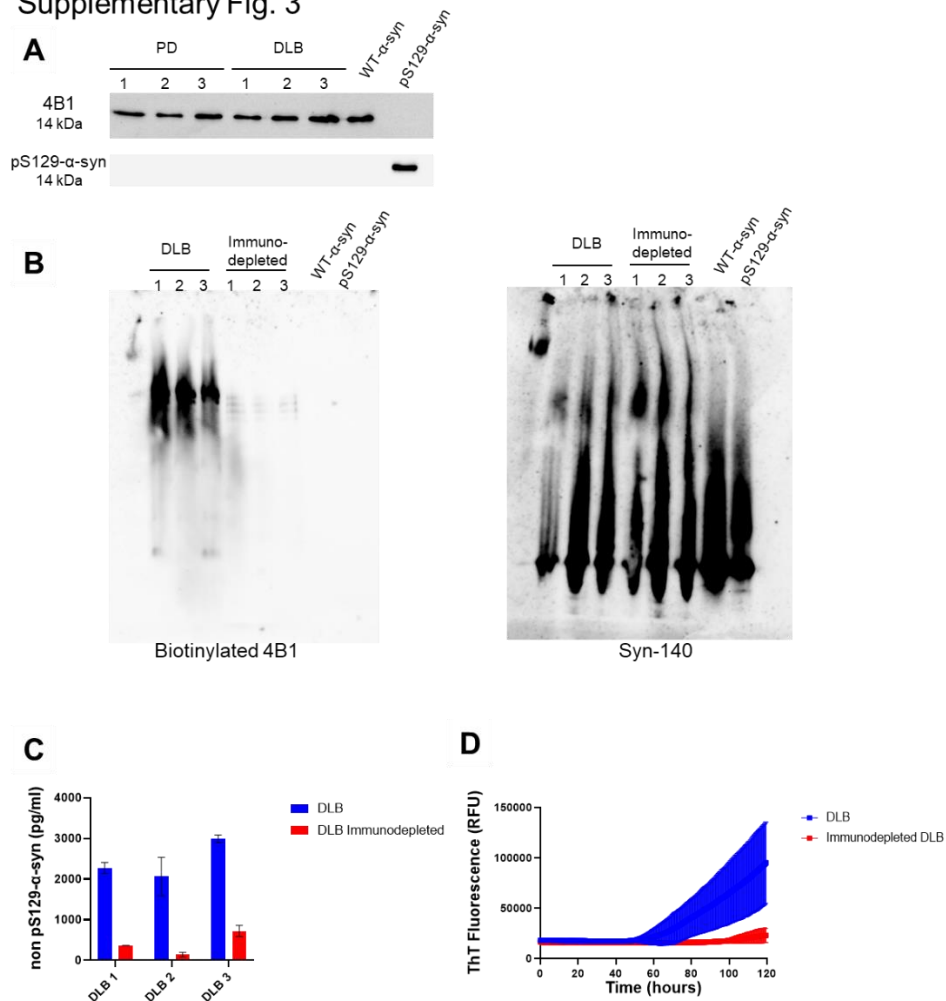
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Supplementary Figure 2. Effect of phosphorylation buffer constituents (pS129-α-syn buffer) on α-syn aggregation, α-syn seeding aggregation, and nucleation-dependent RT-QuIC assay. **A.** Monomeric α-syn was incubated in the presence of various percentages (0, 5, 20, and 50%) of pS129-α-syn buffer (final concentration 100 μM) for 8 days at 37°C with continuous shaking. Fibril formation was evaluated by Th-S fluorescence. The assay was performed in triplicates, and the means ± standard deviations are shown. **B.** Monomeric WT-α-syn (100 μM) was incubated alone, or with 2 μM (final concentration) of WT-α-syn PFFs for 48 hours in presence or absence of pS129-α-syn buffer. Fibril formation was evaluated by Th-S fluorescence. The assay was performed in triplicates, and the means ± standard deviations are shown. **C.** RT-QuIC assay was performed using recombinant monomeric WT-α-syn with or without pS129-α-syn buffer used as substrates for DLB frontal lysates.

Supplementary Fig. 3



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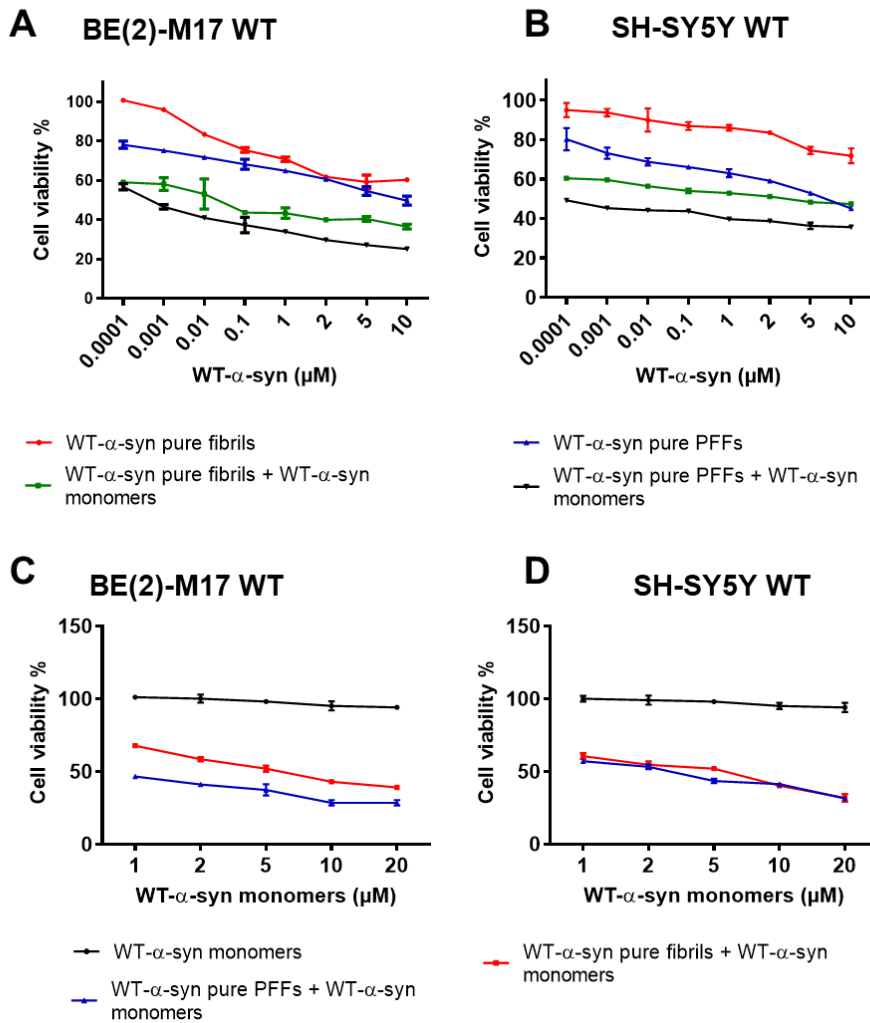
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Supplementary Figure 3. Effect of immunodepleting of WT-α-syn by 4B1 in DLB brain lysates. **A.** Western blot analysis of pS129 (Abcam ab51253) and WT-α-syn in brain lysates from TBS fractions of PD and DLB cases. **B,C.** Native gel analysis and oligomeric ELISA showing immunodepletion of WT-α-syn by 4B1 in TBS fractions of brain lysates from three DLB cases. **D.** RT-QuIC assay using recombinant monomeric WT-α-syn on TBS fractions of brain lysates from three DLB cases and their corresponding immunodepleted samples.

Supplementary Fig. 4



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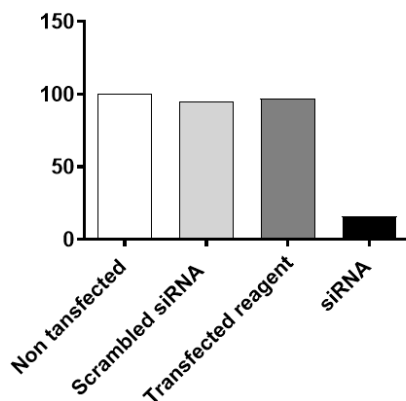
Supplementary Figure 4. The effect of α -syn seeding on the viability of neuroblastoma cells. The viability of BE(2)-M17 and SHSY-5Y WT human neuroblastoma cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). **A.** BE(2)-M17 and **B.** SHSY-5Y WT cells were treated with different concentrations of α -syn pure fibrils or pure PFFs (0.0001-10 μ M) and one hour after treatment, monomeric α -syn to a final concentration of 10 μ M of was added. **C.** BE(2)-M17 and **D.** SHSY-5Y cells were treated with 2 μ M (final concentration) of α -syn pure fibrils or pure PFFs and one hour after treatment, monomeric α -syn was added to a final concentration ranging between 1-20 μ M. The assay was performed in triplicates and the means \pm standard deviation are shown.

Supplementary Fig. 5

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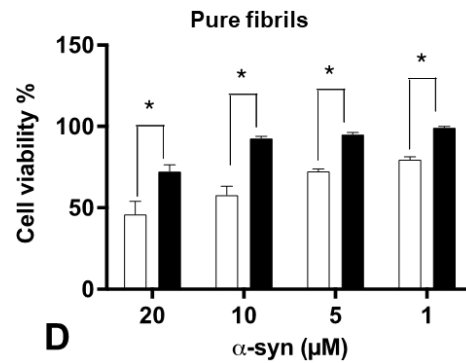


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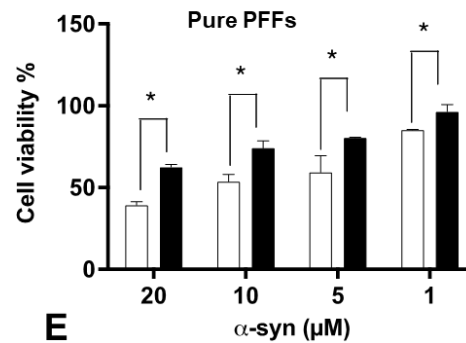


□ Non- transfected cells
 ■ siRNA transfected cells

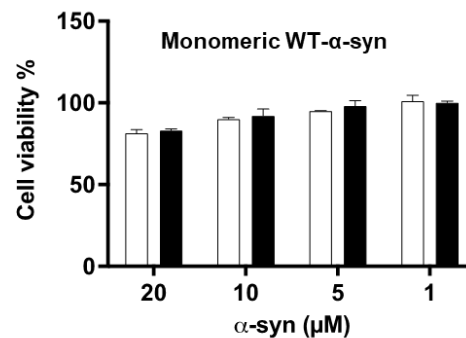
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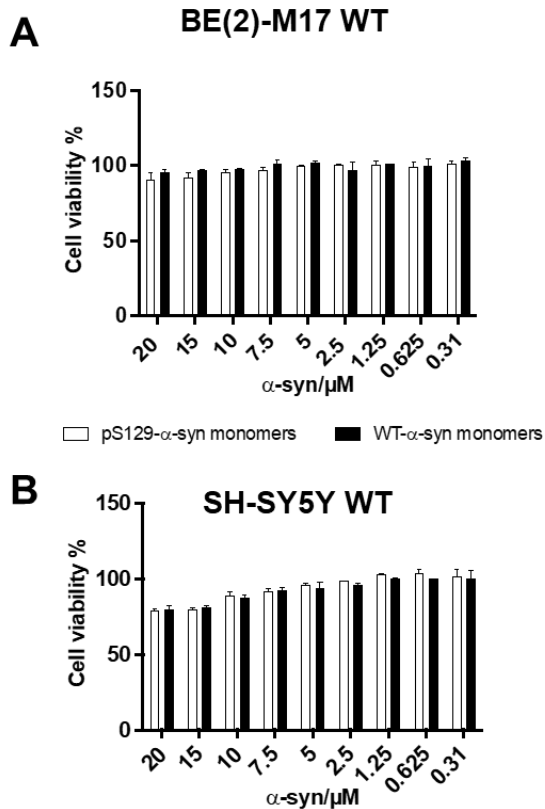
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Supplementary Figure 5. The effect of pure PFFs and fibrils on neuroblastoma cells with knocked down endogenous α -syn. The cell viability of BE(2)-M17 cells, whose endogenous α -syn has been knocked down was studied by the MTT assay. Pre-designed siRNA sequence targeting human WT- α -syn was used to silence the expression of α -syn, and non-targeting scrambled siRNA was used as negative control. **A.** Immunoblotting of cell lysates for total α -syn detection using the mouse monoclonal anti- α -syn (211) antibody and β -actin as loading control. **B.** Quantification of α -syn expression levels by densitometric analysis using ImageJ software. **C-E.** The viability of BE(2)-M17 WT cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). **C.** BE(2)-M17 WT cells were treated with different concentrations of α -syn pure fibrils **D.** pure PFFs. **E.** monomeric WT- α -syn. The assay was performed in triplicates and the means \pm standard deviation are shown. (*, $p < 0.05$).

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Supplementary Fig. 6



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331 **Supplementary Figure 6. The effect of monomeric pS129- α -syn on the viability**
332 **of neuroblastoma cells.** The effect of monomeric WT- and pS129- α -syn on the
333 viability of **A.** BE(2)-M17-WT and, **B.** SHSY-5Y human neuroblastoma WT cells was
334 estimated by the MTT assay. The results are expressed as the percentage of the
335 control average (*i.e.*, untreated cells). The assay was performed in triplicates and the
336 means \pm standard deviation are shown.

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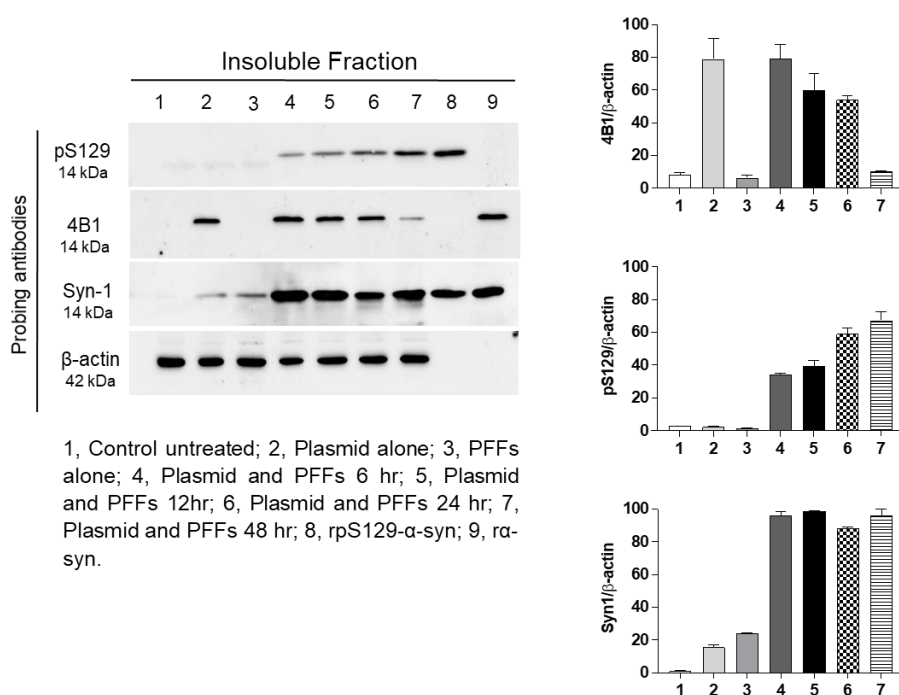
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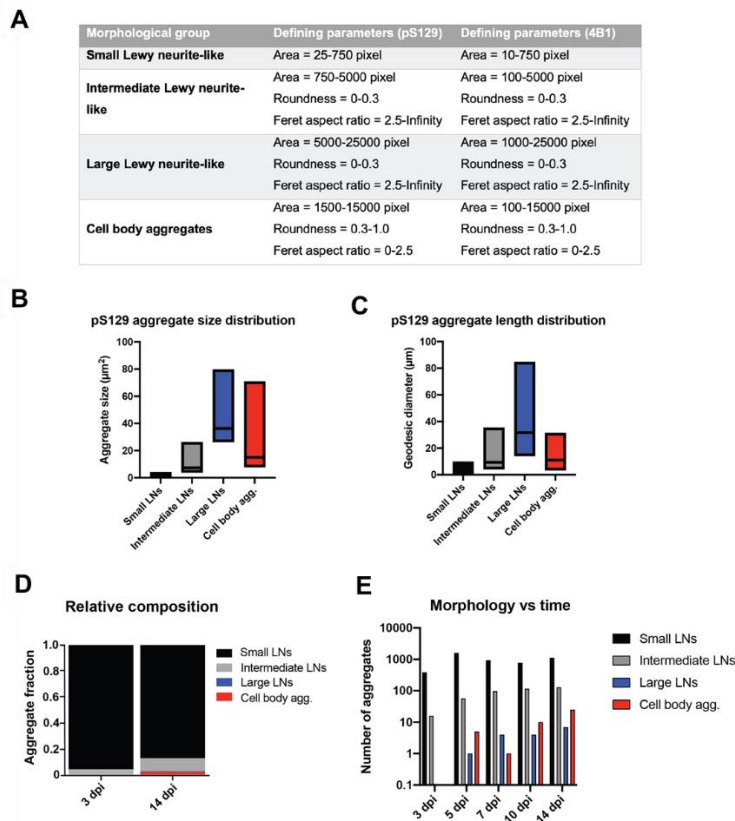
Supplementary Fig. 7



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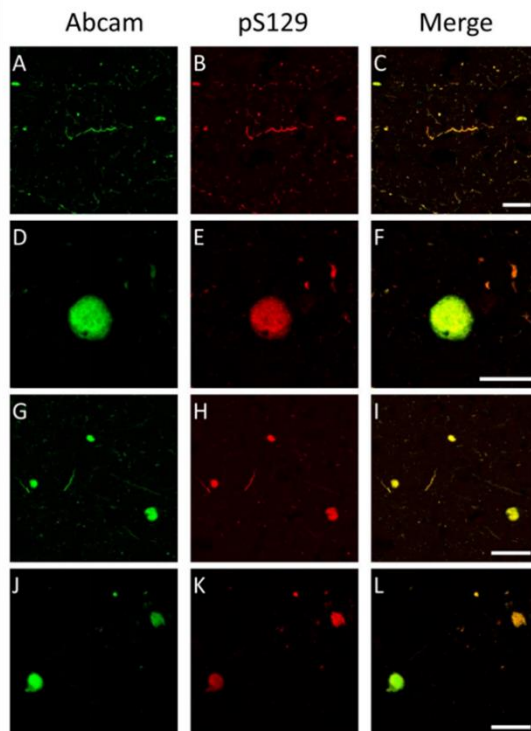
Supplementary Figure 7. Effects of S129A-α-syn seeding on aggregation and accumulation of insoluble pS129-α-syn in a HEK cell model. 10µg of insoluble proteins from cell lysates of untransfected (control) and transfected HEK cells were immunoblotted proteins using antibodies specific to pS129-α-syn (ab51253, Abcam), and total α-syn (Syn-1) at time points 6, 12, 24, 48 hours post PFFs transfection. Recombinant pS129-α-syn (rpS129-α-syn) and recombinant α-syn (rα-syn) proteins were loaded (50ng) as positive controls. Re-immunoblotting with β-actin antibody was performed to normalize the amount of loaded proteins.

Supplementary Fig. 8



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 386 **Supplementary Figure 8. Parameters and characteristics of aggregate subtypes**
 387 **in organotypic slice culture model.** **A.** Defining parameters for morphological
 388 stratification in OHSC experiment. Aggregates were initially defined by pS129-staining
 389 and assigned to one of the groups. Subsequently, 4B1-positive aggregate proportions
 390 were defined inside the pS129-selections. To account for the possibility of lower
 391 proportions of 4B1 in the aggregates, minimum area was set lower than for the
 392 corresponding pS129 area threshold. **B.** Size distribution in μm^2 within each category
 393 of aggregates, defined by their pS129-staining. Ranges were approximately: 0.1-4 μm^2
 394 (small LNs), 4-26 μm^2 (intermediate LNs), 26-80 μm^2 (large LNs), and 8-70 μm^2 (cell
 395 body inclusions). For all groups, the mean aggregate size was in the lower part of the
 396 range of that group. **C.** Corresponding approximate aggregate length (defined by the
 397 geodesic diameter) in μm for each of the four categories. Some overlap in aggregate
 398 length is seen between small, intermediate and large LNs, indicating that while some
 399 aggregates are long and slim, others are shorter and wider. Ranges were
 400 approximately: 0.1-9.7 μm (small LNs), 4.4-35.2 μm (intermediate LNs), 14.2-84.6 μm
 401 (large LNs), and 3.5-31.2 μm (cell body inclusions). **D.** Relative composition of
 402 aggregates by morphology at time points 3 and 14 dpi, displayed as fractions of the
 403 total number of aggregates. At 3 dpi, approximately 4% of the aggregates were
 404 intermediate-sized LNs, while the remaining 96% were small LNs. At 14 dpi, small LNs
 405 constituted around 87.5% of the aggregates, while 10% were intermediate-sized LNs,
 406 0.5% were large LNs, and 2% were cell body inclusions. **E.** Aggregate composition
 407 over time in actual numbers show an initial increase in number of small LNs from 3 to
 408 5 dpi, after which the number only varies slightly. In contrast, the numbers of
 409 intermediate-sized LNs, large LNs and cell body aggregates continue to increase with
 410 time.
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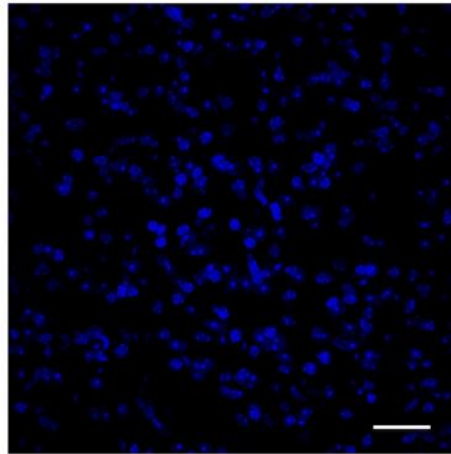
Supplementary Fig. 9



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Supplementary Figure 9. Immunofluorescent staining demonstrating co-localization of Abcam ab51253 pS129 antibody with the in-house pS129 antibody in the amygdala. Scale bars = 30 μm (A-C), 20 μm (D-F; J-L), 50 μm (G-I).

Ipsilateral Striatum at 2WPI (DAPI/Goat anti mouse Alexa 488)



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Supplementary Figure 10. Immunofluorescence analysis of negative control for 4B1 in the ipsilateral striatum following incubation with goat anti mouse Alexa 488 alone, at 2 weeks post injection (40x magnification, Scale bar 50 μ m).

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