

Neurodegeneration and Neuroinflammation are Linked, but Independent of α -Synuclein Inclusions, in a Seeding/Spreading Mouse Model of Parkinson's Disease

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1 **Neurodegeneration and neuroinflammation are linked, but independent of α -synuclein**
2 **inclusions, in a seeding/spreading mouse model of Parkinson's disease**

3

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23

24

25 **Abstract**

26 **Background:** A key process of neurodegeneration in Parkinson's disease (PD) is the
27 transneuronal spreading of α -synuclein. Alpha-synuclein is a presynaptic protein that is
28 implicated in the pathogenesis of PD and other synucleinopathies, where it forms, upon
29 intracellular aggregation, pathological inclusions. Other hallmarks of PD include
30 neurodegeneration and microgliosis in susceptible brain regions. Whether it is primarily
31 transneuronal spreading of α -synuclein particles, inclusion formation, or other mechanisms,
32 such as inflammation, that cause neurodegeneration in PD is unclear.

33 **Methods:** We used spreading/aggregation of α -synuclein induced by intracerebral injection
34 of α -synuclein preformed fibrils into the mouse brain to address this question. We performed
35 quantitative histological analysis for α -synuclein inclusions, neurodegeneration, and
36 microgliosis in different brain regions, and a gene expression profiling of the ventral
37 midbrain, at two different timepoints after disease induction.

38 **Results:** We observed significant neurodegeneration and microgliosis in brain regions not
39 only with, but also without α -synuclein inclusions. We also observed prominent microgliosis
40 in injured brain regions that did not correlate with neurodegeneration nor with inclusion load.
41 In longitudinal gene expression profiling experiments, we observed early and unique
42 alterations linked to microglial mediated inflammation that preceded neurodegeneration,
43 indicating an active role of microglia in inducing neurodegeneration. Our observations
44 indicate that α -synuclein inclusion formation is not the major driver in the early phases of
45 PD-like neurodegeneration, but that diffusible, oligomeric α -synuclein species, which induce
46 unusual microglial reactivity, play a key role in this process.

47 **Conclusion:** Our findings uncover new features of α -synuclein induced pathologies, in
48 particular microgliosis, and point to the necessity of a broader view of the process of "prion-
49 like spreading" of that protein.

50

51 **Key words:** alpha-synuclein, Parkinson's disease, prion-like spreading, microgliosis,
52 transcriptional profiling, neurodegeneration

53

54 **Background**

55 Protein misfolding and aggregation are central pathological processes in neurodegenerative
56 diseases, where they are believed to play a key role in driving the pathology [1, 2]. Proteins
57 such as the amyloid beta peptide (A β) and tau in Alzheimer's disease (AD), TAR DNA-
58 binding protein 43 (TDP43) in fronto-temporal dementia or motor neuron disease, prion in
59 Creutzfeldt-Jakob disease, and finally alpha-synuclein (α -syn) in Parkinson's disease (PD),
60 are all examples of physiologically occurring proteins that, upon pathological misfolding,
61 form oligomers, fibrils, and extracellular (A β , prion) or intracellular (TDP43, tau, α -syn)
62 deposits, and injure neurons in the process [3-5].

63 An important property of these disease-associated proteins is their ability to self-propagate.
64 This has been known for decades in prion diseases, in which disease-associated misfolding
65 proteins themselves are sufficient to induce the disease process when transposed into a
66 susceptible recipient host already decades ago [3, 6]. They do so by acting as a seed and
67 corrupting the endogenous form of the protein, leading it to aggregate and form, over time,
68 intracellular inclusions along interconnected neuronal pathways [7, 8]. Currently, the
69 predominant narrative of this "spreading hypothesis" is that misfolded/aggregated particles of
70 a disease protein move transsynaptically from neuron to neuron, causing dysfunction and
71 damage along the way [4, 8]. Major support for this hypothesis comes from two observations.
72 First, the neuropathological studies by Braak and colleagues, staging tau inclusions in AD
73 [9], and Lewy inclusions in PD [10], suggest a progressive appearance, starting first in a
74 population of susceptible neurons, of proteinaceous intraneuronal inclusions, a process that

75 takes place over decades. Second, postmortem studies of in PD patients that had received
76 intrastriatal fetal neuron transplants to combat dopamine loss, revealed Lewy bodies in a
77 subset of the grafted neurons, indicating a spreading of abnormal α -syn from the diseased
78 neurons of the recipient to those of the donor [11, 12]. Alpha-syn is a presynaptic protein that
79 normally is involved in the regulation of the synaptic vesicle cycle [13, 14]. Its involvement
80 in PD was discovered when it was identified as an essential component of a PD pathological
81 hallmark, the Lewy body [15], and when mutations in its gene, as well as dupli-or triplication
82 thereof, were shown to lead to hereditary forms of the disease [16, 17]. Its prion-like
83 spreading properties have been demonstrated in *in vitro* and *in vivo* model systems, using
84 intracranial injection of Lewy-body containing brain extracts, of viral-construct mediated α -
85 syn overexpression and/or administration of pre-formed fibrils (PFFs) from recombinant α -
86 syn as seeds to induce spreading and aggregation[18] [19-22].

87 The role of α -syn spreading and inclusion formation in PD pathogenesis is still unclear, since no
88 correlation between PD symptoms and α -syn inclusion load was consistently found [23-26].
89 Different possibilities that could explain what ultimately causes neuronal dysfunction and
90 injury and, hence, neurological symptoms, have recently surfaced [27, 28]. Among those, the
91 notion that smaller moieties, or oligomers, of misfolded proteins rather than more fibrillar,
92 deposited forms of α -syn are the most neurotoxic has gained traction [29, 30]. It is important
93 to note that, in the field of AD, it was thought for decades that the A β deposited into plaques
94 is the most harmful to neurons, whereas more recent evidence points to diffusible, soluble
95 small A β moieties as being the major neurotoxic form [31]. In the PD field though, this
96 notion is still debated: while some studies report the potential harm α -syn oligomers can
97 cause [30, 32], other studies still contend that neuronal dysfunction and injury cannot occur
98 without the demonstrable presence of inclusions [33, 34]. In this study, we addressed this
99 issue in the using a α -syn seeding/spreading induced in wildtype mice [20]. We used

100 intracranial administration of recombinant murine α -syn PFFs to induce α -syn spreading and
101 inclusion formation in the brain of wildtype mice, and examined neurodegeneration and
102 microgliosis in brain regions with α -syn inclusions and, importantly, in those without. We
103 observed neurodegeneration in both cases, indicating that neuronal injury can occur
104 independently of the progressive formation of α -syn inclusions. Because neuroinflammation
105 has emerged as a key player in neurodegenerative disease [35], and because microglia are the
106 main cellular effectors of this process [36, 37], we also measured microgliosis in our model.
107 We noticed, in regions with or without inclusions, a surprisingly strong microgliosis (4-5x
108 over baseline), which far surpassed that observed after administration of neurotoxins such as
109 the dopaminergic lesioning agent 6-hydroxydopamine (6-OHDA). In contrast to mice
110 injected with 6-OHDA, neurodegeneration and microgliosis did not correlate with each other
111 in the brains of α -syn PFFs injected mice. Moreover, by measuring gene expression profiles
112 after intrastriatal α -syn PFF injection, we observed numerous significant changes in
113 inflammation-related genes and pathways, and an unusual microglial molecular activation
114 profile that preceded neuron loss and indicated a direct involvement of these cells in the
115 neurodegeneration process. These findings indicated that, in this model, microgliosis does
116 not occur primarily as a response to neuronal damage, but is likely part of an intrinsic
117 response to a process that is independent of the progression of α -syn inclusion formation.
118 Our results demonstrate that PD-like neurodegeneration can occur independently of the
119 presence of α -syn inclusions, and thus that PD-like pathologies is more than just the
120 progressive formation of pathological inclusions. It may involve spreading of other, more
121 soluble forms of toxic aggregates, such as oligomers, inducing an excessive microglial
122 response, before inclusions are formed. We believe these results add an important aspect on
123 how the pathogenic properties of “prion-like” α -syn should be viewed, and how future
124 therapeutic interventions for PD will be designed.

125

126 **Methods**

127

128 *Expression, and purification of recombinant murine α -Syn, and generation of pre-formed*
129 *fibrils (PFFs), and of oligomers*

130 Expression and purification of recombinant murine α -syn and generation of PFFs were
131 performed as described [38]. PFFs were stored aliquoted at -80°C until use. For the
132 preparation of oligomers, recombinant α -syn was purchased from Analytik Jena (Jena,
133 Germany). Oligomers were generated as described [39, 40] by incubating soluble α -syn in 10
134 mM Tris-HCl, 100 mM NaCl under continuous shaking in an Eppendorf Thermomixer at
135 650rpm and 37°C for 24 h, then stored aliquoted at 2 mg/ml at -80°C until use.

136

137 *Western Blot of α -syn PFFs and oligomers*

138 The composition of α -syn PFFs and oligomers was checked by non-denaturing Western Blot.
139 Three different concentrations of oligomers of PFFs (10 ng, 100 ng, 500 ng), were loaded on
140 4-10% Precast Gel Mini Protean TGX (BioRad) according to manufacturer's instructions. To
141 reveal α -syn bands, anti-synuclein antibody clone 4D6 (Covance) was used at 1:2000 dilution
142 (2 h at RT incubation), followed by IRDye^R 800 CW donkey anti-mouse, diluted 1:10.000 (1
143 h at RT incubation). Image was captured with a LI-COR Bioscience C-Digit
144 Chemoluminescence scanner.

145

146 *Animals*

147 Three- to 6- month-old C57Bl/6J mice were purchased from Jackson via Charles River (Bois-
148 des-Oncins, France), or Janvier Labs (Le-Genet-St.-Isle, France). Mice were housed in
149 individually -ventilated cages (IVC) in a conventional animal facility of the University of

150 Luxembourg, or in the facility of SynAging, in Vandœuvre-les-Nancy, France. All animal
151 studies were in agreement with the requirements of the EU Directive 2010/63/EU and
152 Commission recommendation 2007/526/EC. Male or female mice were housed under a 12h-
153 12h dark/light cycle with *ad libitum* access to water and food (#2016, Harlan, Horst, NL).
154 Animal studies were approved by the institutional Animal Experimentation Ethics Committee
155 of the University of Luxembourg and the responsible Luxembourg government authorities
156 (Ministry of Health, Ministry of Agriculture). Alternatively, experiments done at the
157 SynAging site were approved by ethics committee “Comité d’Ethique Lorrain en Matière
158 d’Expérimentation Animale”, and by the governmental agency the “Direction
159 Départementale de la Protection des Populations de Meurthe et Moselle- Domaine
160 Expérimentation Animale”.

161 *Intrastriatal injections of α -syn PFFs, α -syn oligomers, and 6-hydroxydopamine*

162 Alpha-syn PFFs were sonicated in a sonicating waterbath (Branson 2510, Danbury, CT) for 2
163 hours at RT, keeping the temperature constant at 25°C by adding ice as needed, or using the
164 Bioruptor UCD 300 (Diagenode, Seraing, Belgium) with 30 cycles of 15sec ON/ 15sec OFF
165 at 4°C. Sonicated PFFs were kept on ice and used within ten hours. Mice were injected under
166 isoflurane anesthesia (2%) on a heating pad. A 1 cm long mid-line scalp incision was made
167 into the disinfected surgical area and a 0.5 mm hole drilled unilaterally into the skull using
168 stereotaxic coordinates for striatum according to the Mouse Brain Atlas of Franklin and
169 Paxinos [41]. Ten μ g of PFFs, or respective PBS solution (control mice) were administered,
170 in volumes of 2 μ l, within the right dorsal striatum at the following relative-to-bregma
171 coordinates: anterior +0.5 mm, lateral +2.1 mm; depth +3.2 mm. The 24-gauge blunt tip
172 needle of the Hamilton syringe (7105KH, Bonaduz, CH) was inserted down 3.3 mm for 10
173 seconds to form an injection pocket, and the needle remained in place for 2 minutes before
174 and after the injection procedure. The hole was covered with bonewax (Lukens, Arlington,

175 VA) and the wound closed using 7mm Reflex wound clips (Fine Science Tools, Heidelberg,
176 Germany). Two % xylocaine gel was applied to the wound, and mice were allowed to recover
177 from anesthesia before being put back into their home cages. The day of injection of PFFs
178 was named day 0. Same coordinates and similar procedure were used for 6-OHDA or α -syn
179 oligomers. Striatal injection of 6-OHDA has been described elsewhere [42]. Striatal
180 injections of α -syn oligomers were done with 4 μ g oligomers in 2 μ l vehicle. Control mice
181 received the same volume of vehicle (see above). Mice were euthanized in a deep anaesthesia
182 (i.p. injection of Medetomidin, 1mg/kg and Ketamin, 100mg/kg) by transcardial transfusion
183 with PBS. PFF-injected mice were euthanized either at day 13 (13 dpi) or at day 90 (90 dpi)
184 after intrastriatal injections (“day 0”: day of injection). Mice injected with oligomers or with
185 6-OHDA were euthanised at 13 dpi.

186

187 *Tissue extraction and preparation*

188 For immunohistochemistry, extracted brains were fixed in in 4% buffered PFA for 48h and
189 kept in PBS with 0.1% NaN₃ until they were cut with a vibratome (VT1000 S from Leica)
190 into sagittal 50 μ m free-floating sections. Before the staining procedure, sections were kept at
191 -20°C in a cryoprotectant medium (1:1 v/v PBS/Ethylene Glycol, 10g.L⁻¹ Polyvinyl
192 Pyrrolidone). Alternatively, for dopamine measurement or RNA extraction, after removal
193 from the skull, brains were dissected on ice into regions, and isolated striatum and ventral
194 midbrain were quickly weighted, then snap-frozen on dry ice until further processing.
195 Extraction and measurement of striatal dopamine (DA) has been described elsewhere [43].
196 Briefly, after homogenization and derivatization, striatal metabolites were measured with a
197 gas-chromatography/mass-spectrometry set-up (Agilent 7890B GC – Agilent 5977A MSD,
198 Santa Clara, CA). Absolute level of DA were determined using an internal standard, 2-(3,4-
199 Dihydroxyphenyl)ethyl-1,1,2,2-d₄-amine HCl (D-1540, C/D/N isotopes, Pointe-Claire,

200 Canada). For RNA extraction from the ventral midbrain, the RNEasy Universal Kit
201 (Quiagen) was used, after homogenization of midbrain tissues in a Retsch MM 400 device (2
202 min at 22Hz, Haan, Germany). RNA concentrations and integrity were determined using a
203 Nanodrop 2000c (Thermo Scientific) and a BioAnalyzer 2100 (Agilent), respectively.
204 Purified RNAs were considered of sufficient quality if their RNA Integrity Number (RIN)
205 was above 8.5, their 260/230 absorbance ratio > 1, and their 260/280 absorbance ratio = 2.

206

207 *Single and double-label immunohistochemistry*

208 Immunostaining procedures followed standard protocols, as described [44, 45]. All stainings,
209 except those for proteinase-K resistant α -syn inclusions (see below), were performed on free-
210 floating 50 μ m-thick sections. Supplemental Table 1 lists all primary and secondary
211 antibodies used in this study, as well as their dilutions. All other reagents were from Sigma
212 unless indicated otherwise. All antibody incubations were at room temperature, except for the
213 anti-synaptophysin antibody, which was incubated at 4°C. Sections were washed 3 x in PBS
214 between each incubation step. To block endogenous peroxidases and for permeabilization,
215 sections were incubated with 3% H₂O₂ v/v and 1.5% Triton X100 v/v for 30 min. For
216 immunoperoxidase staining with anti-synuclein antibody, this step was followed by an epitope
217 unmasking step with 75% v/v formic acid for 5 min. To avoid unspecific antibody binding,
218 sections were incubated with 5% serum (Vector Laboratories, Burlingame, CA) or 5% BSA
219 w/v in PBS for 1h before they were incubated with the respective primary antibody, or
220 antibodies in case of double labeling. The following day, sections were incubated with a
221 secondary antibody for 1-2 hours (fluorophore-coupled for immunofluorescence, or
222 biotinylated for immunoperoxidase). Singly or doubly fluorescently-stained sections were
223 mounted on Superfrost plus slides (Thermoscientific, Walham, MA), air-dried, and
224 coverslipped using ProLong Gold antifade mountant (Life technologies, Darmstadt,

225 Germany). For immunoperoxidase staining, antibody binding was visualized using an ABC
226 Vectastain Kit (Vector Laboratories), followed by detection with diaminobenzidine (Merck)
227 and H₂O₂ as peroxidase substrates. Sections were mounted, dried overnight and coverslipped
228 with Neo-mount (Merck) after soaking in Neo-clear xylene substitute (Merck) for 10 min.
229 Visualization of Proteinase-K resistant inclusions was done on thin paraffin sections mounted
230 on nitrocellulose (“PET”-blot) as described [46].

231

232 *Proximity ligation assay*

233 Protocol for PLA was adapted to be performed on free floating sections. All reactants were
234 prepared according manufacturer’s recommendations (Duolink, Sigma) and incubation times
235 were as described [47]. Washes were performed in 24- well plates at RT, and reactions
236 volumes were 40μL at 37°C. First, 20μg of anti-pSER129- α-syn mouse monoclonal 11E5
237 antibody (Prothena Biosciences, see suppl. table 1) were conjugated with either plus or minus
238 oligonucleotide probes according manufacturer’s recommendations, and stored 4°C until use.
239 Free floating sections were washed in PBS and permeabilized as described above. Blocking
240 was performed with DuoLink blocking solution for 2h RT. Sections were incubated overnight
241 with both plus and minus probe-linked antibody (1:1 1/750 in Duolink antibody diluent
242 solution). For ligation of the probes, after washing of the probe-linked antibodies (2 X 5min
243 in Duolink’s Buffer A), the ligation-ligase solution was added and incubated for 30 min at
244 37°C. For detection, after washing of the ligation-ligase solution (2 X 5min in Duolink’s
245 Buffer A), sections were incubated with the amplification-polymerase solution for 2.5h at
246 37°C. Sections were then washed in Buffer B for ten minutes, and in Buffer B 0.01X for one
247 minute prior mounting, dried in the dark, and coverslipped. Z-stacks of pictures were
248 acquired at 40X with a Zeiss LabA1 microscope, a maximum intensity projection was created
249 using the Zen Blue 2012 software (Zeiss).

250

251 *Quantitative neuropathology on immunostained sections*

252 Imaging of peroxidase- labelled sections for pSER129- α -syn, and of fluorescently labelled
253 sections for Tyrosine-Hydroxylase (TH), Dopamine Transporter (DAT), or Ionized calcium
254 binding adaptor molecule 1 (Iba1), was done on a Zeiss LabA1 microscope, coupled to a
255 Zeiss Axiocam MRm3 digital camera, and to a PC using the Zeiss Zen Blue 2012 software.

256 Alpha-syn inclusions were visualized by immunostaining for pSER129- α -syn. For the
257 quantitation of α -syn inclusions in the frontal cortex and the amygdala (basolateral nucleus),
258 2 immunoperoxidase labeled (see above) sections/animal were imaged, using the 10x
259 objective (frontal cortex) or the 20x objective (amygdala). A total of 4-6 images were
260 collected for each region (10x objective, 2 x 1.52 mm each image), and digitized. After
261 manually drawing regions of interests and thresholding, the percent image area occupied by
262 immunopositive structures was determined using the ImageJ v. 1.45 (NIH, Bethesda, MD)
263 public domain software. All values obtained from sections of the same animal were averaged.

264 For the quantitation of α -syn inclusions in the SN, double immunostainings for TH and
265 pSER129- α -syn were performed. TH staining was used to locate the SN, and images were
266 acquired at 10x magnification. Percent overlap of pSER129- α -syn signal within the TH
267 immunopositive area was calculated using ImageJ. For the quantitation of synaptophysin-
268 positive synaptic terminals, fluorescently stained sections from each animal were viewed by a
269 Zeiss LSM 710 laser-scanning confocal microscope, using a 20x objective and a software
270 magnification zoom factor was used to obtain images of 180 \times 180 μ m each. For each animal,
271 a total of 4-6 images were collected from the frontal cortex, and 4 from the hippocampal
272 pyramidal region. Images were then transferred to a PC personal computer, and average
273 intensity of positive presynaptic terminals was quantified for each image using the ImageJ.
274 Values from individual animals were averaged. This method to quantify synaptic integrity

275 has been validated by electron microscope quantitation of synaptic densities in a previous
276 study [44].

277 The quantitation of degeneration of TH positive neurons in the SN has been described, and
278 results obtained with this approach have been shown to correlate with stereological cell
279 counts (supplemental material in Ashrafi et al. (2017) [42]). For the quantitation of striatal
280 TH-positive neuronal fibers and of DAT-positive synaptic terminal, doubly labelled sections
281 with anti-TH and anti-DAT were used. A total of six to nine 40x pictures (223.8 x 167.7 μ m
282 each) of the dorsal striatum, from 2-3 sections per mouse, were acquired using the optical
283 sectioning system Apotome.2 (Zeiss). The percent area occupied by TH and DAT was
284 determined using Image J software and averaged for each mouse.

285 For the quantitation of microglial activation in the hippocampus and frontal cortex, 2 sections
286 /animal were labeled for the microglial marker *Iba1*. For the frontal cortex, a total of 6, and,
287 for the hippocampus, a total of 3-4 images were collected with a 40x objective (223.8 x 167.7
288 μ m each image). Digitized images were transferred to a laptop computer, and, with ImageJ v.
289 1.45, after thresholding, average area occupied by *Iba1*-positive microglia was measured. All
290 values obtained from sections of the same animal were averaged. For the quantitation of the
291 microglial activation in the SN, TH and *Iba1*- double-labeled sections were imaged with a
292 10x objective. Average area covered by TH-positive neurons in control mice was used to
293 determine the region of interest, restricted to the SN, to measure microglial activation. Four
294 subregions of the SN were imaged and quantified for each mouse [42]. *Iba1* immunopositive
295 cells were quantified within each subregions, averaged for each of them, and converted in
296 mm². For each mouse, the sum of the four averaged subregions was used as a measure for
297 microglial activation.

298 All quantitative neuropathological analyses were performed blinded on coded sections, and,
299 for each of the measurements, codes were only broken when quantification for that measure

300 in all animals was complete. For all measures, the ipsilateral and contralateral values of PBS-
301 injected control mice were similar (no statistical difference detected), these values were
302 grouped. Statistics on quantitative histological data were done using the GraphPad Prism 8
303 software. ANOVA followed by Dunnett's post-hoc was used for normally distributed data
304 sets. Pearson's test, or Spearman's rank test where appropriate, were used to study linear
305 correlations. P values smaller than 5% were considered as significant.

306

307 *Microarray analysis and calculation of differentially expressed genes*

308 GeneChip Mouse Gene 2.0ST Arrays (Affymetrix) were used for transcriptional profiling.
309 Total RNAs (150ng) were processed using the Affymetrix GeneChip® WT PLUS Reagent
310 Kit according to the manufacturer's instructions (Manual Target Preparation for GeneChip®
311 Whole Transcript (WT) Expression Arrays P/N 703174 Rev. 2). In this procedure, adapted
312 from Bougnaud et al. (2016) [48], the purified, sense-strand cDNA is fragmented by uracil-
313 DNA glycosylase (UDG) and apurinic/aprimidinic endonuclease 1 (APE 1) at the unnatural
314 dUTP residues and breaks the DNA strand. The fragmented cDNA was labelled by terminal
315 deoxynucleotidyl transferase (TdT) using the Affymetrix proprietary DNA Labelling Reagent
316 that is covalently linked to biotin; 5.5 µg of single-stranded cDNA are required for
317 fragmentation and labelling, then 3.5 µg of labeled DNA + hybridization controls were
318 injected into an Affymetrix cartridge. Microarrays were then incubated in the Affymetrix
319 Oven with rotation at 60 rpm for 16 hr at 45°C, then the arrays were washed and scanned
320 with the Affymetrix® GeneChip® Scanner 3000, based on the following protocol:
321 UserGuide GeneChip® Expression Wash, Stain and Scan for Cartridge Arrays P/N 702731
322 Rev. 4, which generated the Affymetrix raw data CEL files containing hybridization raw
323 signal intensities were imported into the Partek GS software. First, probe intensities were
324 summarized to gene expression signals using Partek default options (GCcontent adjustment,

325 RMA background correction, quantile normalization, log₂ transformation and summarization
326 by means).

327 For statistical analysis, the normalized and log₂ transformed data was loaded into the
328 R/Bioconductor statistical environment. The rank product (Package: **RankProd**) approach
329 was chosen to determine the differentially expressed genes (DEGs) [49-51]. Rank product
330 statistics were computed, since they have been shown to enable a robust non-parametric
331 analysis of microarray datasets with limited number of samples [49]. Estimated p-values and
332 pfp (percentage of false prediction) values were determined and used as nominal and
333 adjusted significance scores, respectively. Pfp scores estimate the significance of differential
334 expression after adjusting for multiple hypothesis testing, and can have values larger than 1.
335 The chosen significance cut-offs were p-value < 0.05 and pfp < 0.1. A relaxed cut-off (pfp <
336 0.1 instead of < 0.05) was chosen to avoid loss of information for the subsequent enrichment
337 analysis, which combines several genes below this threshold to enable detection of pathway
338 alterations. No minimal fold change threshold was applied.

339 For visualization of differential gene expression, Venn diagrams and heatmaps were
340 generated using the **VennDiagram** and **gplots** packages, respectively, in R. Data pre-
341 processing included removal of all transcripts missing gene IDs and duplicated entries (after
342 ranking). To avoid mismatches or other discrepancies, the Affymetrix probe sets IDs were
343 used to calculate the overlapping DEGs between the different comparisons. Diagrams were
344 generated for the following criteria and comparisons: 1) 13 dpi & 90 dpi - ipsiPFF *versus*
345 ipsiPBS (p-value < 0.05); 2) 13 dpi & 90 dpi – ipsiPFF *versus* ipsiPBS (pfp < 0.1); 3) 13 dpi
346 & 90 dpi - ipsiPFF *versus* contraPFF (p-value < 0.05); 4) 13 dpi & 90 dpi – ipsiPFF vs
347 contraPFF (pfp < 0.1). In a second step, we were interested in investigating the expression
348 direction of the overlapping transcripts between the early to late timepoint. Therefore, we
349 extracted the probe IDs, matched the individual lists and grouped them into high and low

350 expressed transcripts. Then, the above mentioned venn diagrams were generated with these
351 newly generated lists. Heatmaps were generated using the *heatmap.2* function for ipsiPFF vs
352 ipsiPBS and ipsiPFF vs contraPFF for p-value < 0.05 and pfp < 0.1 at 13 dpi and 90 dpi
353 respectively. The log₂-transformed data matrix was used to plot. Additionally, we applied
354 hierarchical top down clustering (*cor* and *hclust* basic R functions) and the data matrix was
355 scaled row-by-row generating Z-scores.

356

357 *Gene Set Enrichment Analysis*

358 The enrichment analysis for GO terms (biological processes (BP) only) was performed using
359 the GUI (graphical user interface) version GSEA (version 3.0) published by the Broad
360 Institute (download: <http://software.broadinstitute.org/gsea/downloads.jsp>) [52]. All
361 parameters were set to default in GSEA, except “Collapse dataset to gene symbols” was set to
362 “false”, “Permutation type” was set to “gene_set” and “Max size: excluding larger sets” was
363 set to “250”. One optimization step was introduced: a customized GMT/GMX file was
364 generated in R/Bioconductor with mouse NCBI EntryzIDs as gene identifiers. This file was
365 used as the “Gene sets database” in GSEA. The resulting enrichment scores (ES) were
366 obtained applying the weighted Kolmogorov-Smirnov-like statistics. ES reflect the level to
367 which a gene set is overrepresented among the top up- or down-regulated genes in a ranked
368 gene list, then, the ES statistic was normalized (normalized enrichment scores, NES) as
369 described [52]. Finally, the p-value significance scores were adjusted for multiple hypothesis
370 testing using the method by Benjamini and Hochberg[53] to provide final FDR scores. A
371 network map of the enrichment analysis results was generated using Cytoscape [54]. The
372 mapping parameters used in Cytoscape were: p-value < 0.05, FDR Q-value < 0.1 (default
373 setting is 1) and Overlap > 0.5. The enrichment map was automatically launched from GSEA
374 and created in Cytoscape. In the enrichment maps, nodes represent enriched gene sets

375 associated with BPs, and edges the degree of similarity between them using the overlap
376 coefficient (threshold > 0.5). Further curation of gene sets was done manually. Since gene
377 sets with similar gene compositions tend to group together, such gene set clusters were easily
378 identifiable. Nodes grouped into more than one gene cluster according to this procedure were
379 assigned to the most overlapping cluster, i.e. the cluster they were associated with by a
380 shorter sequence of connecting edges in the ontology graph. All softwares used are listed in
381 Supplemental table 2.

382

383 *Identification of cellular source of DEGs*

384 For the identification of the cellular source of specific DEGs, the public database
385 <https://www.brainrnaseq.org/> was used.

386

387 **Results**

388

389 **Western blot characterisation of α -syn moieties**

390 A non-denaturing blot of the α -syn moieties used in this study is shown in supplemental Fig,
391 1. For intrastriatal injections, α -syn oligomers were used non-sonicated, whereas α -syn PFFs
392 were sonicated. Based on their profile on WB, the oligomer preparation was composed
393 mainly of monomers, dimers, and trimers, as well as higher molecular weight species. The
394 sonicated PFFs were composed mainly of monomers and dimers, and higher molecular
395 weight species. When compared to their non-sonicated counterparts, sonicated PFFs seemed
396 to have less of all these components, consistent with a shearing effect of sonication that
397 produces smaller α -syn fragments, which may then act as seeds.

398

399 **Intrastriatal injection of PFFs causes bilateral α -syn inclusions in multiple brain regions**

400 Because we wanted to capture the early features of α -syn spreading associated pathologies,
401 we decided to focus our investigations on time points when, these pathologies have not
402 reached a peak yet [20]. Since α -syn inclusions have been suggested to be a major driver of
403 PD-like pathology [34, 55], we first looked at the appearance of these inclusions in our
404 model.

405 To determine if intrastriatal injection of murine α -syn PFF reliably induced propagation of
406 fibrillar α -syn in our mice, we first performed immunohistochemistry against pSER129- α -
407 syn on sections of both brain hemispheres 13 days and 90 days after they had been injected
408 with PFFs (13 dpi, 90 dpi). Immunostaining for pSER129- α -syn is the most commonly used
409 approach to detect α -syn inclusions in rodent or human brain tissues [56].

410 At an early time point after α -syn PFF administration (13 dpi), we only detected few
411 pSER129- α -syn positive inclusions in frontal cortex, amygdala, and SN, and more in the
412 ipsilateral striatum (supplemental Fig. 2).

413 However, at 90dpi, we observed robust appearance of pSER129- α -syn positive cellular and
414 neuritic inclusions in the ipsi- and contralaterally, in the same brain regions (Fig.1 A).
415 Quantitation of image area occupied revealed median coverage of 10% for the ipsilateral
416 frontal cortex, 5% for the contralateral frontal cortex, 8% for the ipsilateral amygdala, 4.2%
417 for the contralateral amygdala, and 12% for the ipsilateral SN. No or very few α -syn
418 inclusions were found in the contralateral SN and striatum, and no inclusions in either side of
419 the hippocampus. Cells containing inclusions had neuronal morphology. In the ipsilateral SN,
420 fluorescent double staining for pSER129- α -syn and TH, a marker for dopaminergic neurons
421 in the SN, showed that 85% of inclusions co-localized with TH-positive neurons, indicating
422 that most, if not all, inclusions are localized in neurons.

423 To determine if the pSER129- α -syn positive inclusions were composed of fibrillated
424 filaments, we performed a Proteinase-K assay on paraffin-embedded tissue sections (PET

425 assay) [46]. We observed numerous pSER129- α -syn positive inclusions in these tissue
426 sections (Fig. 1 B), indicating that most inclusions were proteinase-K resistant.
427 Inclusions are not the only α -syn species that have been suggested to be linked to
428 neurodegeneration in PD. To determine if regions without detectable α -syn inclusions, such
429 as the hippocampus, were still affected by abnormal α -syn after injection of PFFs, we
430 performed a Proximity Ligation Assay (PLA). This assay has been used for detecting
431 oligomeric forms of α -syn in human [57] and mouse models of PD [58]. We observed greatly
432 enhanced signal intensity in the hippocampi of PFF-injected mice than in those of control
433 mice (Fig. 1C), indicating the presence of abnormal levels of oligomeric α -syn in that area.
434 Overall, the pattern of α -syn inclusions we observed 90 dpi matches that described at a
435 similar time point by Luk et al. (2012) [20]. The robust appearance of intracellular α -syn
436 inclusions in this model opened up the possibility of analyzing how they are associated with
437 other pathological hallmarks, such as neurodegeneration and α -inflammation, and which of
438 these events might precede the others.

439

440 **Intrastriatal injection of PFFs causes bilateral synaptic loss and unilateral** 441 **dopaminergic neuron injury that is independent of α -syn inclusions**

442 We set out to determine to what extent the presence of neuronal α -syn deposition was linked
443 to neurodegeneration, 90 dpi after intrastriatal administration of PFFs.

444 First, we analyzed synaptic degeneration in the hippocampus and frontal cortex. In these
445 brain regions, we measured the level of the presynaptic protein synaptophysin.
446 Synaptophysin is a good marker for synaptic integrity [44, 59, 60], and pathological synaptic
447 alterations have been reported in PD post-mortem tissues [61]. Roughly 60% of PD patients
448 suffer from cognitive impairments and dementia [62], indicating that their hippocampus, as a
449 major region involved in memory formation, and their higher cortical association areas are

450 affected. Finally, *in vitro*, addition to PFFs to cultured primary hippocampal neurons was
451 reported to affect these neuron's synaptic integrity and function [63]. Thus, we measured
452 synaptophysin ipsi- and contralaterally in these brain regions in mice 90 dpi after PFF
453 administration (Fig. 2). We found, in both regions, a highly significant, bilateral 20-25%
454 reduction of this protein. Interestingly, we noticed this decrease in the absence of α -syn
455 inclusions in the hippocampus. The α -syn oligomers though (Fig. 1) in that region may be
456 linked to synaptophysin loss.

457 Next, we examined the SN, because it contains dopaminergic neurons that are one of the
458 most susceptible to PD-associated disease challenges. We measured the area occupied by
459 tyrosine hydroxylase (TH)-positive neuronal profiles in the SN ipsilaterally, where α -syn
460 inclusions were present (see above), but also contralaterally, which was without such
461 inclusions. We did not find any sign of degeneration in the striatum or SN at 13 dpi
462 (Supplemental fig. 2). We found though a 16% decrease of TH-positive neurons in the
463 ipsilateral SN, that was significant, but not in the contralateral SN (Fig 2). To determine if
464 striatal axonal projections of dopaminergic neurons were affected in our model, we analyzed
465 the morphological integrity of these projections and their synaptic terminals. We observed, 90
466 dpi, a significant decrease in TH-positive axonal fibers as well as in dopamine transporter
467 (DAT) positive synaptic terminals, in the ipsilateral, but not the contralateral striatum. To
468 confirm ipsilateral striatal injury, we measured the neurotransmitter dopamine (DA) in
469 dissected ipsi- and contralateral striata of PFF-injected and PBS control mice (n=8-12/group).
470 We found a significant decrease in ipsilateral striatum of PFF mice compared their ipsilateral
471 PBS controls (19.5 \pm 5.8 versus 27.5 \pm 7.3 pmol/mg; p=0.02 by ANOVA followed by
472 Sidak's posthoc, results are means \pm S.D.), but no difference between contralateral striatum
473 of PFF mice compared to their compared their ipsilateral PBS controls (27.3 \pm 3.7 pmol/mg
474 versus 28.5 \pm 5.7 pmol/mg). This conformed our histological observations.

475

476 **Widespread, pronounced, and bilateral microgliosis, caused by intrastriatal injection of**
477 **α -syn fibrils.**

478 Microglia, the local CNS innate immune defense cells [64], react rapidly to CNS infection or
479 injury in an orchestrated fashion. Functional imbalances of these cells can precipitate disease
480 outcomes [36, 37, 65]. While strong microgliosis has been reported in PD and models thereof
481 [66-68], the role of these cells in disease initiation and progression of PD is poorly
482 understood.

483 To better understand the role of these cells in the context of α -syn spreading, and more
484 precisely to determine if they have a role in driving the neurodegeneration we observed, we
485 first analysed their response using a specific marker (Iba1), in mouse brains after injection of
486 α -syn PFFs. We observed a surprisingly strong (4-5 times over control in some brain
487 regions), microgliosis in brain regions with (bilaterally in frontal cortex, amygdala, SN) at 90
488 dpi. The microgliosis was present in brain regions with inclusions, but also those without
489 (hippocampus) or very little (contralateral SN) inclusions (Fig. 3). While no significant Iba1
490 increase was seen at 90 dpi in the ipsilateral striatum in PFF injected mice, microglial
491 Cluster-of-Differentiation 68 (CD68), a marker for phagocytic activity, was increased. No
492 significant microgliosis was observed at 13 dpi (Supplemental fig. 2). Microglia in PFF-
493 injected mice had thickened, though still ramified, processes, and an intensely stained cell
494 soma. In the cerebellum, which was devoid of α -syn deposits in all mice, we could not detect
495 any differences in Iba1 positive microglia between PFF-injected and control PBS injected
496 mice (not shown).

497 Our observations indicate that a robust, widespread microglial reaction is an important part of
498 the α -syn spreading process, and warranted further investigation into the pathological
499 implications of that reaction.

500

501 **Neurodegeneration and microgliosis neither correlated with α -syn deposition, nor with**
502 **each other.**

503 To gain insight into the pathological properties of α -syn inclusions, we correlated the
504 inclusion load with neurodegeneration and with microgliosis measured locally in frontal
505 cortex and SN. We found that inclusion load correlated with neither of the two (Fig. 4). Thus,
506 neurodegeneration as well as microgliosis induced by α -syn PFFs were independent of α -syn
507 deposition.

508 The strong microgliosis in different brain regions after administration of PFFs prompted us to
509 look into this observation further. In the brain, microglia react rapidly to tissue injury to
510 control the damage and clear up cell debris [37, 69]. Thus, microglial reaction is typically
511 secondary to an underlying neurodegenerative process, and, as a consequence, increase of
512 microglial reaction is directly associated with increase in neuronal damage. For instance, we
513 have observed that microglial reaction (measured on Iba1 immunostained sections) correlated
514 with TH neuron loss in the ipsilateral SN after unilateral 6-OHDA lesioning (Fig. 4B2), and
515 with synapse or dendritic loss in the cortex after lesioning with the excitotoxin kainic acid
516 [70]. After intracerebral injection of α -syn PFF though, we found that microglial reaction was
517 not only much stronger than after injection of neurotoxins (4-5x *versus* 2-3x over control),
518 but also failed to correlate with measures of neurodegeneration (TH neuron loss in the SN,
519 synaptophysin in the cortex and hippocampus) (Fig. 4B). This observation indicates that the
520 microglial reaction to α -syn spreading may be a direct response to factors produced during
521 that process, and not just a secondary response to neuronal degeneration, as is the case after
522 injection of neurotoxins.

523

524 **Microglia across several brain regions react strongly to intrastriatal injection of α -syn**
525 **oligomers**

526 Several studies have indicated that microglia are activated *in vitro* by α -syn oligomers [71,
527 72]. As described above, we have observed the presence of α -syn oligomers, notably in the
528 hippocampus, after intrastriatal injection of α -syn PFFs. To test whether α -syn oligomers
529 could be the factor that leads to a strong microglial reaction during the α -syn spreading
530 process, we injected such oligomers into the same location as the PFFs, the dorsal striatum.
531 Just 13 dpi, we observed, on Iba1 stained sections, a strong microglial reaction in the
532 ipsilateral striatum, frontal cortex, and hippocampus (Fig. 5). Qualitatively, the reaction was
533 even stronger than 90 dpi after PFF injection. This observation shows that, in a mouse brain,
534 α -syn oligomers can induce a microglial reaction, even at a distance from the injection site.
535 Thus, α -syn oligomers emerge as the likely factor that, by diffusing through the brain,
536 induces a strong microglial reaction during the process of α -syn spreading.

537

538 **Transcriptional profiling of ventral midbrain reveals most gene expression changes**
539 **occur 13 days after α -syn PFF injection**

540 To investigate the molecular underpinnings of the neurodegeneration and of the microglial
541 response accompanying α -syn spreading, we generated a gene expression profile from ventral
542 midbrain of PFF injected and control mice using the Affymetrix gene expression profiling
543 platform. Because microglial response typically starts early after an insult [64, 73], we
544 analyzed the midbrain gene expression profiles 13 dpi (no neurodegeneration) and 90 dpi
545 (neurodegeneration in the ipsilateral striatum and midbrain) after intrastriatal α -syn PFF
546 injection.

547 We focused on two comparisons of ventral midbrain gene expression profiles: 1. ipsilateral
548 midbrain of PFF-injected mice (ipsi PFF, with degeneration of nigral TH neurons and their
549 striatal projections) *versus* ipsilateral midbrain of control PBS-injected mice (ipsi PBS), 2.
550 ipsilateral midbrain of PFF-injected mice *versus* contralateral midbrain of the same, PFF-
551 injected, mice (contra PFF, without loss of nigral TH neurons and their striatal projections).
552 We figured that these two comparisons would be best suited to reveal relevant gene
553 expression changes.

554 The number of DEGs that emerged in these comparisons, and the number of overlapping
555 DEGs between the two time points (13 dpi and 90 dpi) are shown in Fig. 6. By comparing
556 ipsi PFF to ipsi PBS, applying a cut-off of $p < 0.05$, we found a total of 2.631 significant DEG
557 at 13 dpi, and significant 2584 DEG at 90 dpi, with 985 overlapping DEGs between the two
558 time points. After correcting for multiple hypothesis testing at a cut-off of $p < 0.1$, we
559 found 266 DEGs at 13 dpi, and 82 DEGs at 90 dpi, with 39 DEGs overlapping between the
560 two times points. The majority of overlapping DEGs showed upregulation at 13 dpi, but
561 downregulation at 90 dpi (Venn diagrams of overlapping DEGs in Fig. 7), indicating active
562 gene expression at the early time point after PFF injection. By comparing ipsi PFF to contra
563 PFF, we found 3.477 significantly DEGs at 13 dpi, and 3.209 DEGs at 90 dpi, with 1356
564 overlapping DEGs between the two time points. At $p < 0.1$, we found 648 DEGs at 13 dpi,
565 and 588 DEGs at 90 dpi, with 227 overlapping DEGs. At 13 dpi, we found a similar number
566 of upregulated *versus* downregulated DEGs, but at 90 dpi, we saw that most DEGs were,
567 interestingly, downregulated.

568 Taken together, these two comparisons indicate that enhanced gene expression changes
569 occurred in the ventral midbrains of both hemispheres at 13 dpi, probably setting the stage for
570 the subsequent pathological events. In contrast, at 90 dpi, in the ipsilateral midbrain, most
571 DEGs dial their expression level back, indicating a reduction in gene transcription, while the

572 major pathological events now appear to take place at the protein level, and are measurable
573 with quantitative histology (see above).

574

575 **Gene set enrichment revealed early involvement of inflammation in the α -syn**
576 **seeding/spreading process**

577 To investigate which molecular pathways underlie the α -syn spreading process and its
578 associated pathologies, in particular microgliosis, we generated an enrichment map, using
579 Gene-Set Enrichment Analysis (GSEA, see Methods), with each gene set based on a
580 Biological Process (BP, see Methods for details). To obtain a global view of the BPs
581 alterations during the evolution of α -syn spreading induced pathologies, we used manual
582 curation to group gene sets into biologically meaningful gene clusters associated with high
583 order pathological processes (Fig. 7).

584 Our first observation was that, in ipsilateral midbrains of PFF-injected mice compared to
585 those of PBS-injected ones, 261 BPs were enriched at 13 dpi, but, surprisingly none at 90 dpi.
586 In contrast, we observed that, at 90 dpi, all BPs in ipsilateral midbrains of PFF-injected mice
587 *versus* those of PBS-injected ones (total of 1067 BPs), showed reduced gene activity. This
588 observation indicates a significant shift from enhanced to greatly reduced transcriptional
589 activity in the time frame between 13 dpi and 90 dpi, and confirms the observations on DEGs
590 depicted in Fig. 6.

591 We then observed that many gene clusters with enhanced transcriptional activity at 13 dpi in
592 ipsi PFF were associated with inflammation/immune processes (Fig. 7, upper panels), while
593 gene clusters associated with similar activities had reduced transcriptional activity at 90 dpi,
594 in particular compared to ipsi PBS (Fig. 7, lower left panel). This indicated that, after an
595 initially enhanced activity of genes regulating inflammation/immune responses, that activity
596 was strongly reduced at a stage when pathology was detectable histologically.

597 Another interesting observation we made was that some gene clusters containing BPs
598 associated with reduced gene activity at 90 dpi, were related to dopaminergic neuron activity
599 (e.g. catecholamine/dopamine metabolic processes, locomotor behavior, regulation of
600 synaptic transmission regulation of signaling pathways upon growth factor stimulus). The
601 reduced gene activity in midbrain dopaminergic neurons was likely a reflection of their
602 pathological demise.

603 Taken together, these observations point to an important role for inflammatory/immune
604 processes, in the initiation and progression of neurodegeneration in the context of α -syn
605 spreading.

606

607 **Gene expression changes confirm early microglial reaction in response to α -syn** 608 **seeding/spreading**

609 To identify the immune cell type(s) active in the inflammatory response to α -syn
610 seeding/spreading, we looked at the 20 most highly changed DEGs and their cellular source
611 for each time points after PFF injection (Fig. 8). We used a public database based on single
612 cell expression profiling from mouse brain to assign a cell type to each DEG in our list (see
613 Methods).

614 At 13 dpi, in both the ipsi PFF *versus* ipsi PBS as well as the ipsi PFF *versus* contra PFF
615 comparison, we observed that the majority of genes with enhanced expression were
616 microglial (ipsi PFF *versus* ipsi PBS: 9 out of 20, or 45%, ipsi PFF *versus* contra PFF: 8 out
617 of 20, or 40%). This indicates a strong gene expression activity of these cells, well before
618 morphological changes can be detected histologically.

619 In contrast, at 90 dpi, we observed that only 1 out of 20 (5%) of DEGs was microglial in both
620 comparisons (ipsi PFF *versus* ipsi PBS, ipsi PFF *versus* contra PFF). The majority (50%) of
621 DEGs in the ipsi PFF *versus* ipsi PBS comparison were neuronal.

622 The observation that the majority of DEGs at 13 dpi were microglial, confirmed an early and
623 strong response of these cells, at least on the molecular level.

624

625 **Unusual microglial molecular signature, induced by intrastriatal injection of α -syn** 626 **PFFs, precedes neurodegeneration**

627 To better understand the microglial molecular processes accompanying the α -syn spreading
628 process, we looked at expression of a series of genes coding for factors associated with
629 typical pro-inflammatory (M1) or anti-inflammatory (M2) profile [74], with the same
630 comparison pairs as before: ventral midbrains ipsi PFF *versus* ipsi PBS, and ventral
631 midbrains ipsi PFF *versus* contra PFF (Table 1). In our model, we observed no clear-cut pro-
632 inflammatory M1 nor anti-inflammatory M2-profile. Interestingly, we also observed no
633 evidence, at 13 dpi or 90 dpi, for gene expression changes in classical M1 markers such as
634 *Il1b*, *Tnfa*, or *Nos2*. M1 markers whose gene expression was enhanced were *Cybb*, *Ptgs2*, and
635 *Cxcl10*. NADPH oxidase 2 (Nox2), coded by *Cybb*, generates free oxygen radicals, which
636 can harm neurons [75]. Cyclooxygenase 2 (Cox2), coded by *Ptgs2*, generates arachidonic
637 acid metabolites, some of which have been reported to be neurotoxic [76] or form neurotoxic
638 dopamine-quinone adducts [77]. Thus, this may be mediators of the neurodegeneration at 90
639 dpi observed in ipsi PFF midbrains. The only M2 marker that showed enhanced gene
640 expression was *Mrc1*, coding for the mannose receptor. Other microglial activation markers
641 that have not been associated specifically with an M1 or M2 profile though, such as *Cd68*,
642 *Tyrobd*, *Trem2*, *Tlr2*, *P2ry6*, and *Aif1*, showed increased expression at 13 dpi and/or 90 dpi.

643 *Mrc1*, *Cd68*, *P2ry6*, *Aif1* gene products are all involved in phagocytic processes and/or signal
644 transduction [78-80]. We had observed CD68 upregulation by immunostaining in the striatal
645 projection area of dopaminergic neurons (Fig. 3). The *Tlr2* gene product is a receptor for α -
646 syn, an interaction that elicits the production of microglial neurotoxins [81], and anti-TLR2
647 antibody administration has been reported to have therapeutic efficacy in mouse models of α -
648 syn toxicity [82]. *Tyrobp* and the gene for its receptor, *Trem2*, whose product is involved,
649 among other processes in the regulation of microglial phagocytosis [83], were also
650 upregulated.

651 Finally, to see if there was an astroglial and peripheral immune cell involvement in our
652 model, we listed gene expression data for typical markers of these cells from our gene
653 expression dataset (Supplemental table 3). Enhanced expression of a series of astroglial genes
654 in ipsi PFF midbrain indicates a reaction of these cells. Enhanced expression of *Ptprc*, which
655 codes for CD45, a marker that can be both expressed by microglia and invading
656 macrophages, and of *Cd4*, which codes for the helper T cell antigen CD4, in the same region,
657 indicates possible infiltration of peripheral immune cells that could contribute to neuronal
658 injury [84-86]. Overall, we conclude that this unique molecular signature in the ipsilateral
659 ventral midbrain at 13 dpi underlies the initial molecular events that lead to the
660 neurodegeneration we observed at 90 dpi. Since neurodegeneration in the contralateral SN
661 has been reported at later time points after PFF injection previously [20], in one study even in
662 the absence of α -syn inclusions [87], it is reasonable to assume that, at a point past 13dpi, the
663 same molecular signature occurs there.

664 Our data indicate that inflammatory events, in particular those associated with microglia, and
665 not inclusion formation, are initiators of neurodegeneration in the context of α -syn spreading
666 in PD.

667

668 **Discussion**

669 In this study, we have used a seeding/spreading model of α -syn, based on striatal injection of
670 PFFs in the mouse brain, to investigate key questions on how two major pathological features
671 of PD, α -syn inclusion formation and neuroinflammation, contribute to neurodegeneration.
672 We provide evidence that: 1. α -syn inclusion formation does not correlate with
673 neurodegeneration: in areas with inclusions, the inclusion load did not correlate with the
674 extent of neurodegeneration, and, in at least one area (hippocampus), neurodegeneration was
675 detected in the absence of inclusions; 2. an exceptionally strong microglial response was seen
676 across different brain regions, but this response did also not correlate with neurodegeneration;
677 3. the most likely driver of the microglial response were diffusible α -syn oligomers; 4. gene
678 expression changes indicative of early neuroinflammatory events in the ventral midbrain, in
679 particular in microglia, appeared before nigro-striatal degeneration, and some of these factors
680 could be the driver for downstream neurodegeneration. Our study provides novel insights into
681 underlying pathological processes of α -syn spreading mediated PD-like neuronal injury.
682 We undertook this study because it is unclear how different pathological processes relate to
683 each other in PD. In particular, it is intensely debated whether the α -syn spreading and
684 inclusion formation are the main driving forces in disease initiation and progression, or
685 whether other processes do this or at least participate in them [88-90]. While the nature of the
686 initial trigger of α -syn's misfolding and seeding is still unknown, the hypothesis that its
687 spreading and seeding in a "prion-like" fashion along interconnected neuronal pathways,
688 ultimately leading to inclusions, is a major driver of the PD pathological process has gained
689 momentum [8, 91]. Observations on PD patients have provided indirect evidence for that
690 view. For instance, the Braak hypothesis [10] posits that α -syn pathology starts in lower
691 motor nuclei of the brainstem (such as the Dorsal Motor Nucleus of the Vagus) or even in the
692 PNS, then gradually moves upwards and, in doing so, causes various PD symptoms, from

693 early non-motor to later motor and cognitive and psychiatric ones, to appear. Other routes of
694 propagation, such as starting and spreading out from the olfactory bulb, have also been
695 suggested [8]. Such a gradual progression of disease could elegantly be explained by α -syn
696 spreading like a prion. It was also observed that fetal grafts of dopaminergic neurons into the
697 striatum of PD patients develop α -syn inclusions after a few decades, which they could have
698 acquired as consequence of α -syn spreading from the surrounding disease tissue [12]. More
699 direct evidence for the importance of α -syn spreading in inducing PD-like disease comes
700 from experimental models. In rodent or primate models, direct injection, in different brain
701 regions, of PD brain tissue, isolated Lewy bodies, or PFF made out of recombinant α -syn, or
702 viral vector driven local overexpression of α -syn, induces a variety of PD-related pathologies,
703 including α -syn spreading along connected neurons and inclusion formation [19]. Peripheral
704 PFF injections, such as intramuscular or intestinal, have been reported to also lead to PD-like
705 pathologies in the brain of mice [92-94]. These studies have cemented, experimentally, the
706 process of “prion-like” propagation and inclusion formation of α -syn.

707 The mechanism of this process has been investigated in *in vitro* systems. Cultured neurons
708 secrete as well as take up circulating α -syn, and various underlying mechanisms have been
709 proposed, such as unusual forms of endo- and exocytosis, or nano-tubes [95]. Ingested,
710 presumably misfolded α -syn, corrupts its endogenous counterpart and leads it to form
711 pathological inclusions [55]. During the process, different neuronal functions, such as axonal
712 transport or mitochondrial respiration, get impaired, neurons malfunction and may ultimately
713 die [96, 97]. Glial cells have also been reported to take up α -syn, and, in some cases, this can
714 lead to pathological inclusions as well as in the case for oligodendrocytes in Multiple System
715 Atrophy [98]. The toxic potential of inclusions has also been investigated *in vivo*. In a mouse
716 model of α -syn spreading, where inclusion formation was followed *in vivo* by multi-photon
717 laser microscopy, the formation of intraneuronal inclusion was reported to coincide with

718 neuronal dysfunction [33]. Another study has shown a weak correlation between loss of TH
719 neurons in the SN and a global score of inclusion load after striatal PFF injection in both
720 mice and rats, but a strong correlation between the two measures after direct injection of
721 PFFs into the SN of rats [34]. Thus, it is tempting to conclude that α -syn inclusions are at the
722 very least one major driver of PD pathologies. But a closer look at other evidence reveals
723 several unresolved questions in this otherwise elegant picture. In post-mortem brain tissues of
724 early or late PD, the correlation between α -syn inclusion (Lewy body) load and nigral
725 degeneration is not always clear [25, 26]. Across different studies looking at various brain
726 structures affected in PD, α -syn inclusions have been reported in areas with high, moderate,
727 or no neuronal loss [26]. Some PD patients, including some familial forms, have PD
728 symptoms and loss of nigral neurons without detectable α -syn inclusions [27]. Some neurons,
729 such as GABAergic neurons, while appearing in the path of α -syn spreading, never develop
730 inclusions [27]. Interestingly, one study, comparing Incipient Lewy Body Disease (ILBD) to
731 PD autopsy material, reported that neuronal loss precedes α -syn inclusion formation in the
732 SN [99]. In a rodent model where spreading is driven by viral overexpression of α -syn in the
733 Dorsal Motor Nucleus of the Vagus, while intact neuronal architecture was essential for the
734 spreading process to happen, neurodegeneration and inclusion formation were also found to
735 be independent processes [58].

736 Non-fibrillar forms of misfolded α -syn, notably oligomers, diffusing for long distances within
737 the brain, have been suggested to drive neuronal dysfunction and degeneration [30, 88]. In
738 our study, we indeed found evidence for neurodegeneration that was independent of the
739 presence of inclusions, and, in the hippocampus, even appeared in the complete absence of
740 those, but in the presence of oligomers. Published evidence suggests that the hippocampus
741 remains devoid of α -syn inclusions even 180 days after PFF injection into the striatum [20].
742 Our data therefore does not support the notion of a direct relationship between the formation

743 of α -syn inclusions and neurodegeneration, but rather indicate that the α -syn spreading
744 process may lead to the formation of pathological oligomers that may be the driver of PD-like
745 neurodegeneration.

746 Pathologically misfolded α -syn can drive neuronal injury in PD by different means, and those
747 include mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress and
748 lysosomal dysfunction, disequilibrium in cytosolic Ca^{2+} , neurotoxic oxidized dopamine,
749 disruption of axonal transport, and neuroinflammation [97]. The relative contribution of these
750 different processes to neuronal demise is unclear. Neuroinflammation though has received
751 particular attention because of its widespread involvement in various neurological diseases
752 and the potential for therapeutic modulation [73, 100, 101]. The major cellular mediators of
753 this process are microglia. Microglia are a particular kind of myeloid cells that originate from
754 the yolk sack and populate the nervous system during early stages of development, where
755 they act as the innate, resident immune cells [64, 80]. During development and under normal
756 conditions, they modulate nervous system homeostasis, prune synapses and regulate their
757 formation. Under pathological conditions, they act as the primary line of defense against
758 infectious organisms, and clear endogenous tissue debris after injury [37, 64, 80]. They
759 undergo a substantial morphological and functional transition to activated, or reactive,
760 microglia, which makes them functionally equivalent to macrophages [64]. Evidence
761 suggests though that, in many neurological conditions, they are not only reacting to disease,
762 but play an active part in tissue injury exacerbation and propagation [80]. This pathological
763 process is, in particular in PD, incompletely understood. While microglial activation can be
764 induced by neuronal injury and/or misfolded and aggregated protein, notably α -syn oligomers
765 or fibrils [102], it is still unclear how and when microglial activation damages healthy tissue
766 and exacerbates the neurological disease process. In PD, a strong microgliosis is observed
767 *post mortem* in the SN [67, 103]. Longitudinal imaging studies with PET ligands

768 demonstrated an early microglial activation in various regions beyond the SN, such as cortex,
769 hippocampus, basal ganglia, and pons, but no correlation with other pathological measures,
770 including clinical scores, of PD emerged [104, 105]. Interestingly, in striatal fetal grafts
771 implanted in PD patients [12], microglia activation was observed years before the appearance
772 of α -syn inclusions [106]. In different toxin-induced PD rodent models, microgliosis was
773 reported to precede, coincide, or follow the appearance of neuronal demise [67], while in a
774 transgenic human α -syn model [107] and in rats injected with PFFs into the striatum [108],
775 microgliosis, measured histologically, was shown to precede neurodegeneration.

776 These studies are based on the observation mainly, if not exclusively, of morphological
777 changes of microglial response using immunostaining techniques for generic cell markers.
778 While informative, the detection of morphological changes indicating microglial activation
779 does not yield enough information on the actual physiological or molecular profile of these
780 cells. The common distinction to characterize two functional states of activated microglia is
781 the M1/M2 terminology, with M1 representing a pro-, whereas the M2 representing an anti-
782 inflammatory activation status [74]. This distinction however is often inadequate as microglia
783 commonly have a spectrum of activation states that may change over the course of the
784 disease [109]. Recent gene expression profiling approaches have revealed a bewildering
785 complexity in microglial heterogeneity [36, 110]. Evidence suggests a “core” gene expression
786 profile response that is associated with every neurodegeneration condition, while expression
787 changes of a more restricted set of genes may be specific for each condition, leading to the
788 concept of disease-specific microglial signatures [110]. Our study provides new insights into
789 the molecular underpinnings of neuroinflammation preceding neuronal injury in a PD-like
790 context of α -syn spreading, and points to an active role of microglia in inducing
791 neurodegeneration. First, we show, at the level of gene expression, that neuroinflammation-
792 linked processes are activated, and that many microglial genes had increased expression

793 levels at an early (13 dpi), which then were down-regulated at a later (90 dpi) time point after
794 PFF injection. Microglia genes that code for factors causing neurodegeneration showed
795 increased expression 13 dpi only in the ipsilateral midbrain, where TH loss was observed
796 later, at 90 dpi. Among these were *Cybb*, which codes for NADPH oxidase 2, an enzyme that
797 catalyses the production of tissue harming free radicals [75], and *Ptgs2*, which codes for
798 cyclooxygenase 2 (Cox2), an enzyme that forms prostanoids from arachidonic acid, some of
799 which are neurotoxic [76, 111]. Interestingly, *Tlr2*, *Trem2*, and *Tyrobp* RNAs showed
800 increased levels in our model at 13 dpi. Many genes linked to microglial activation are
801 regulated by *Tyrobp*, a tyrosine kinase binding protein acting that binds to *Trem2*. The
802 *Tyrobp/Trem2* pair triggers pathways that are involved in the inhibition of TLR-mediated
803 inflammation, and in modulating phagocytosis [83]. *Tlr2*-deficient mice are protected against
804 neurodegeneration induced by transgenic α -syn overexpression [112]. In prodromal PD,
805 TLR2 immunoreactivity on microglia was reported to be enhanced, whereas in late stage PD,
806 it wasn't [113], indicating that, just like in our model, the microglial response happens in
807 early phases of the disease and changes over time. Alpha-syn, in particular in its oligomeric
808 form, activates microglia *in vitro* through Toll-like receptors [71, 102], and targeting TLR2
809 by immunotherapy was shown to be beneficial in α -syn pathology models [82].

810 The absence of increased gene expression of common pro-inflammatory mediators such as
811 *Il1b* and *Tnfa* in our α -syn spreading model is puzzling, since these are factors associated
812 with most, if not all, inflammatory conditions. Of note though is that we also did not observe
813 enhanced expression of these factors when primary microglia were exposed to our α -syn
814 PFFs, while they responded strongly to bacterial lipopolysaccharide (not shown). It is
815 possible that the increased expression for these genes was missed and occurs at a time point
816 after PFF injection that we haven't looked at, or that they are indeed not expressed in this
817 model. Overall, the gene expression signature of microglia we detected was neither typical

818 pro-inflammatory M1- nor anti-inflammatory M2-like, and our findings give further credence
819 to the notion that microglia evolve on a spectrum of functional states as the disease
820 progresses.

821

822 **Conclusion:** Taken together, our data indicate that, at least in the initial period of PD-like
823 disease progression that is associated with α -syn spreading, non-deposited pathological forms
824 of α -syn, such as oligomers, drive neurodegeneration in different brain regions *via* their
825 action on microglia. Triggered microglia respond early, before neurodegeneration is apparent,
826 by producing neurotoxic compounds, and through what appears to be a series of different
827 activation states as the disease progresses. Our findings contribute toward first answers to key
828 unresolved questions around neuroinflammation in PD [100], and have important
829 implications for the design of therapeutic interventions during the early stages of the disease.

830

831 **Additional files**

832

833 **Abbreviations**

834 6-OHDA:6-Hydroxydopamine; □13 dpi: 13 days post injection; 90 dpi: 90 days post
835 injection; A□: amyloid beta peptide; AD: Alzheimer's disease; □-syn: alpha-synuclein; BP:
836 biological process; CD68: cluster of differentiation 68; Contra: contralateral; COX2:
837 Cyclooxygenase 2; CNS: central nervous system; DA: dopamine; DAT: dopamine
838 transporter; DEG: differentially expressed gene; ES: enrichment score; FDR: false discovery
839 rate; GO: gene ontology; GSEA: gene set enrichment analysis; Iba1: ionized calcium binding
840 adaptor molecule; Ipsi: ipsilateral; PBS: phosphate-buffered saline; PET assay: paraffin-
841 embedded tissue assay; PFF: Pre-formed fibril; Pfp: percent false positives; PLA: proximity
842 ligation assay

843 p-SER129- α -syn: alpha-synuclein phosphorylated at serine position 129; SN: Substantia
844 Nigra; TDP43: TAR DNA-binding protein 43; TH: tyrosine-hydroxylase; TLR2: Toll-like
845 receptor 2; WB: Western blot.

846

847 *Declarations*

848

849 *Ethics approval*

850 Animal studies performed at the Luxembourg Centre for Systems Biomedicine were
851 approved by the institutional Animal Experimentation Ethics Committee of the University of
852 Luxembourg, and the responsible Luxembourg government authorities (Ministry of Health,
853 Ministry of Agriculture). Alternatively, experiments done at the SynAging site were
854 approved by ethics committee “Comité d’Ethique Lorrain en Matière d’Expérimentation
855 Animale”, and by the governmental agency the “Direction Départementale de la Protection
856 des Populations de Meurthe et Moselle- Domaine Expérimentation Animale”.

857

858 *Consent for publication*

859 All authors have approved of the contents of this manuscript and provided consent for
860 publication.

861

862 *Availability of data and materials*

863 Obtaining α -syn PFFs requires a MTA with Biogen. Obtaining the 11A5 antibody requires a
864 MTA with Prothena Biosciences The original data (Western Blot, quantitative histology) are
865 available from the corresponding author upon reasonable request.

866

867 *Competing interests*

868 AW is currently employed by Biogen. KB is now employed at AbbVie. There are no other
869 competing interests.

870

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876

877 *Authors contributions*

878 P.G., W.W., D.C., and M.B, designed the study. P.G., W.W., O.H., A.Ma., S.B., E.K., T.H.,
879 A.W., C.S., A. Mi., T.P., A.A., N.F. did the experiments (stereotactic surgery, tissue
880 processing, stainings, imaging and RNA extraction). T.K., N.N. generated the microarray
881 data. K.J.S., E.G. analyzed the microarray data. P.G., W.W., O.H., K.J.S., R.B., W.S.-S.,
882 K.B., M.M., M.B analyzed and interpreted the data. M.B. wrote the paper. All authors read
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1222

1223 **Figure and table legends**

1224

1225 **Figure 1.** Intrastriatal injection of murine α -syn PFFs induced α -syn inclusions in various
1226 brain regions. Mice were euthanized 90 days after injection (90 dpi). A. PhosphoSER129 α -
1227 syn immunostaining showed numerous α -syn inclusions in neuritic and neuronal body
1228 structures in different brain regions. Widespread α -syn inclusions were observed bilaterally in
1229 frontal cortex and the amygdala, ipsilaterally in the striatum and the Substantia Nigra (SN),
1230 and only minimally in the contralateral striatum and SN. None were observed in the
1231 hippocampus. No inclusions were observed in either side of the brains of PBS-injected
1232 control mice. Pictures show the ipsilateral side of these mice. B. Proteinase-K digestion on
1233 thin sections generated from paraffin-embedded tissue blocks revealed the presence of
1234 digestion-resistant α -syn inclusions stained for PhosphoSER129 α -syn. Shown here are
1235 ipsilateral striatum and amygdala for illustration. C. Proximity-ligation assay using a
1236 monoclonal PhosphoSER129 α -syn antibody showed the presence of enhanced levels of
1237 oligomeric forms of α -syn in the hippocampus of PFF-injected mice, where no inclusions
1238 could be detected 90 dpi, compared to PBS-injected controls. Scale bar = 250 μ m (A), 250
1239 μ m (B), 25 μ m (C).

1240

1241 **Figure 2.** Intrastriatal injection of murine α -syn PFFs induced neurodegeneration in various
1242 brain regions. Mice were euthanized 90 dpi. In the frontal cortex and the hippocampus, a
1243 significant bilateral loss of synaptophysin-positive presynaptic terminals was observed (first
1244 two rows). In the striatum, a significant ipsilateral loss of TH-positive axonal fibers and
1245 DAT-positive synaptic terminals was observed (3rd and 4th row). In the SN, a significant loss
1246 of TH-positive neurons was observed only ipsilaterally. For group comparisons and graphing,

1247 ipsilateral PBS measures were combined contralateral PBS measures, since they were similar.
1248 Pictures show the ipsilateral side of PBS-injected mice. **** p<0.0001, ** p<0.01, compared
1249 to PBS controls by Dunnett's post-hoc; n = 6-11/group. Scale bars: 18 μ m (for frontal
1250 cortical and hippocampal synaptophysin panels), 22.5 μ m (for striatal TH and DAT panels),
1251 80 μ m (for Subst. Nigra panels)

1252

1253 **Figure 3.** Intrastriatal injection of murine α -syn PFFs induced widespread microgliosis in
1254 different brain regions. Mice were euthanized 90 dpi. Panels show microgliosis measured on
1255 Iba1-stained sections of frontal cortex (upper row), hippocampus (second row), striatum (3rd
1256 row), and Subst. Nigra (last row), and on CD68-stained sections of striatum (4th row). A very
1257 strong microgliosis (up to 4x over control) was observed bilaterally in frontal cortex,
1258 hippocampus, and SN. No increase in Iba1 signal, but a significant bilateral increase in CD68
1259 signal was observed in the striatum of PFF-injected mice. For group comparisons and
1260 graphing, ipsilateral PBS measures were combined contralateral PBS measures, since they
1261 were similar. Pictures show the ipsilateral side of PBS-injected mice. **** p<0.0001, *
1262 p<0.05, compared to PBS controls by Dunnett's post-hoc; n = 6-11/group. Scale bars: 22.5
1263 μ m (for all panels).

1264

1265 **Figure 4.** Different PD-related pathologies in the brains of mice injected intrastrially with
1266 α -syn PFFs do not correlate with each other. Mice were euthanized 90 dpi. A. α -syn
1267 inclusion load did no correlate with neurodegeneration (loss TH-positive neurons, A1) or
1268 with microgliosis (A2) in the SN (Nigra) , nor with neurodegeneration (loss of
1269 synaptophysin-positive synaptic terminals, A3) or with microgliosis (A4) in the frontal
1270 cortex (Cortex). B. Microgliosis did not correlate with loss of TH-positive neurons in the SN
1271 after intrastriatal PFF injection, but did so after intrastriatal injection of the toxin 6-OHDA.

1272 The microgliosis, measured on Iba1-stained section, was also much higher in the Subst. Nigra
1273 of PFF-injected mice than in that of 6-OHDA-injected mice.

1274 All measures shown are from the ipsilateral brain sides; similar observations were made for
1275 the contralateral sides of PFF-injected mice. Correlation analyses were done using Spearman
1276 rank test for data set including α -syn inclusion load measures, and with Pearson's test for
1277 data sets with the other measures.

1278

1279 **Figure 5:** Strong microglial response after intrastriatal injection of α -syn oligomers.
1280 Oligomers were prepared and injections were performed as described in Materials and
1281 Methods. A strong microgliosis was observed in different brain regions 13 dpi, showing that
1282 α -syn oligomers are robust microglial activators in vivo. Scale bar = 40 μ m.

1283

1284 **Figure 6.** Differentially expressed genes induced in the ipsilateral ventral midbrain of mice at
1285 13 dpi (A) and 90 dpi (B) after intrastriatal injections of α -syn PFFs. Comparisons for each
1286 time point were made between gene expressed in ipsilateral ventral midbrain of PFF-injected
1287 mice versus those in the ipsilateral ventral midbrain for PBS-injected mice (ipsi PFF *vs.* ipsi
1288 PBS, n =6/group; upper 4 panels), and between gene expressed in ipsilateral ventral midbrain
1289 *versus* the contralateral ventral midbrains of PFF-injected mice (ipsi PFF *vs.* contra PFF, n
1290 =6/group; lower 4 panels). Panels on the left show Venn diagrams with numbers of DEGs
1291 with a significance level of $p < 0.05$. Because some gene products have an effect within
1292 biological pathways while the expression of their genes may only change minimally, we
1293 show DEGs that emerge with this level of statistical stringency. In ipsi PFF *vs.* ipsi PBS, the
1294 number of DEGs was 2631 at 13 DPI, and 2584 at 90 DPI, with 985 DEGs common to both
1295 time points. In ipsi PFF *vs.* contra PFF, the number of DEGs was 3477 at 13 DPI, and 3209
1296 at 90 DPI, with 1356 DEGs common to both time points. Panels on the right show Venn

1297 diagrams with the number of DEGs after adjusting for multiple hypothesis testing at $p < 0.1$.
1298 In ipsi PFF vs. ipsi PBS, the number of DEGs was 266 at 13 DPI, and 82 at 90 DPI, with 39
1299 DEGs common to both time points. In ipsi PFF vs. contra PFF, the number of DEGs was 648
1300 at 13 DPI, and 588 at 90 DPI, with 227 DEGs common to both time points. The Venn
1301 diagrams below the main ones indicate the number of DEGs that show enhanced (“up”)
1302 versus decreased (“down”), as well as the overlaps, in the different comparisons.

1303

1304 **Figure 7.** Enriched inflammatory pathways precedes neurodegeneration in mouse ventral
1305 midbrains after intrastriatal α -syn PFFs injection. Enrichment map of gene expression
1306 profiles were derived from GSEA. Statistics were done by weighted Kolmogorov-Smirnov,
1307 gene set size limits were set to min15 – max250. Details of curation procedure used to group
1308 BPs (represented as dots, either red if upregulated, or blue if downregulated) into high-level
1309 functional gene set clusters of BPs of related biological function are described in Material &
1310 Methods. At 13 dpi, comparing ipsi PFF to either ipsi PBS or contra PFF, most BPs were
1311 upregulated and associated with gene sets related to immune and inflammation processes.
1312 This shows that, in the ipsilateral nigro-striatum, neuroinflammation precedes
1313 neurodegeneration (measurable at 90 dpi), and might contribute to its development. At 90
1314 dpi, comparing ipsi PFF to ipsi PBS, all BPs, including those associated with inflammation
1315 gene sets, were downregulated, possibly reflecting the neurodegenerative process itself.
1316 Comparing ipsi PFF to contra PFF at this time point, most BPs were upregulated.

1317

1318 **Figure 8.** Top 20 DEGs in mouse ventral midbrain after intrastriatal injection of α -syn PFF
1319 indicate involvement of microglia in initial pathological events. Cellular source of DEGs was
1320 determined using the brain RNAseq database: <https://www.brainrnaseq.org/>. The top panels
1321 show pie charts with the cellular source of the 20 top DEGs when comparing ipsi PFF to ipsi

1322 PBS (left panels) or ipsi PFF with contra PFF (right panels) at 13 dpi and 90 dpi. At 13 dpi,
1323 comparing ipsi PFF with ipsi PBS or contra PFF, 45% and 40%, respectively, of the top 20
1324 DEGs were microglial. At 90 dpi, comparing ipsi PFF with ipsi PBS, 50% of top 20 DEGs
1325 were neuronal, possibly a reflection of neurodegeneration. The bottom panel lists the gene
1326 products of the gene symbols, coded proteins, the associated cell type 20, the fold change
1327 (FC) and the pfp of all top 20 DEGs for each comparison.

1328

1329 **Table 1: Unique molecular features of microglial response to intrastriatal α -syn PFF**
1330 **injection.**

1331 Table shows genes and their coded proteins for factors typically associated with microglial
1332 pro-inflammatory M1 profile (highlighted in yellow), for factors typically associated with
1333 microglial anti-inflammatory M2 profile (highlighted in green), and for generic microglial
1334 activation factors (highlighted in blue). Comparisons are between ventral midbrains of ipsi
1335 PFF *versus* ipsi PBS and of ipsi PFF *versus* contra PFF. The rank p-values and the FDRs are
1336 given. The proteins whose genes have either a significant p-value (<0.05) or pfp < 0.1 are
1337 highlighted in bold. Already at 13 dpi, *Cybb*, the gene for NADPH oxidase 2, a mediator of
1338 oxidative stress, was upregulated in ipsi PFF midbrain, a region that showed
1339 neurodegeneration at 90 dpi (see above). Another gene, *Ptgs2*, whose product,
1340 cyclooxygenase 2, has been reported to generate neurotoxic arachidonic acid metabolites,
1341 was also upregulated in ipsi PFF midbrains, most strongly so (pfp <0.1) when comparing ipsi
1342 PFF with contra PFF. This gene can be expressed also in astrocytes. *Cd68*, whose protein is
1343 linked to lysosomal function and possibly acts as a scavenger receptor, was also strongly
1344 upregulated in ipsi PFF midbrains. Finally, a triad of factors associated with microglia
1345 activation (*Tyrobp*, *Trem2*, *Tlr2*) was upregulated at 13 dpi in ipsi PFF midbrains. Their

1346 downregulation at 90 dpi in ipsi PFF versus ipsi PBS could indicate that the inflammation
1347 process starts to resolve.

1348

1349

1350 **Supplemental figures and tables legends**

1351

1352 **Suppl. fig.1:** Western blot characterization of α -syn moities used for intrastriatal injections.

1353 Loading of the gel was as follows: lanes 1, 6, 14 – ladder; lanes 5, 10,11- blank; lanes 2,3,4 –

1354 α -syn oligomer, 10, 100, and 500 ng respectively; lanes 7, 8, 9 – α -syn PFFs, not sonicated,

1355 10,100, 500 ng respectively; lanes 11, 12, 13 – α -syn PFFs (sonicated), 10,100, 500 ng

1356 respectively. Bands from corresponding to the MW of α -syn monomers, dimers, and trimers

1357 are circled (red, blue, orange, respectively). Note the presence of high molecular weight

1358 moities (visible as a smear) after loading of non-sonicated PFFs. The sonication process of

1359 PFFs appears to reduce all higher molecular weight species of α -syn and the amount of

1360 dimers and monomers, since smears and monomer/dimer bands were visible after loading

1361 100 ng of non-sonicated PFFs, but not after loading the same amount of sonicated PFFs.

1362

1363 **Suppl. fig. 2:** Minimal presence of α -syn inclusions in different brain regions 13 DPI after

1364 intrastriatal injection of α -syn PFFs. Only few α -syn inclusions were seen, ipsi- and

1365 contralaterally, in the frontal cortex, amygdala, and ipsilaterally, but not contralaterally, in

1366 the striatum and SN. Scale bar = 130 μ m.

1367

1368 **Suppl. Fig. 3:** No nigro-striatal degeneration and microgliosis 13 dpi after intrastriatal

1369 injection of α -syn PFFs. No loss of striatal TH-positive axons (first row), striatal DAT-

1370 positive synaptic terminals (second row), nigral TH-positive neurons (third row) was

1371 observed 13 dpi after injection of PFFs. No increase of Iba1-positive microglial reaction in
1372 the SN was observed (last row). Microphotographs show examples of PBS-injected control
1373 brains (ipsilateral) and ipsilateral α -syn PFF-injected brains. Scale bars = 25 μ m (striatal
1374 panels), 200 μ m (Subst. Nigra panels).

1375

1376 **Suppl. fig. 4:** Heatmaps illustrating the ventral midbrain gene expression patterns at 13 dpi
1377 and 90 dpi after intrastriatal injection of α -syn PFFs. Heatmaps are shown for p-values < 0.05
1378 or pfp < 0.1. Scaled row expression values (row Z-score) in red indicate higher, in blue lower,
1379 and in white unchanged gene expression (see Materials and Methods for details).
1380 Comparisons of ipsi PFF *versus* ipsi PBS (upper panel row) and ipsi PBS *versus* contra PFF
1381 (lower panel row) reveal distinctive gene expression patterns at 13 dpi for both comparisons
1382 (whether higher, pfp < 0.1, or lower, p<0.05, statistical stringency is used), whereas a
1383 distinctive patterns at 90 dpi only appears in the ipsi PFF *versus* contra PFF, but not in the
1384 ipsi PFF *versus* ipsi PBS, comparisons.

1385

1386 ***Supplemental tables legends***

1387 **Suppl table 1: Antibodies used in this study**

1388 **Suppl table 2: Softwares used in this study**

1389 **Suppl table 3: Molecular astrocyte and peripheral immune cell markers**

Figures

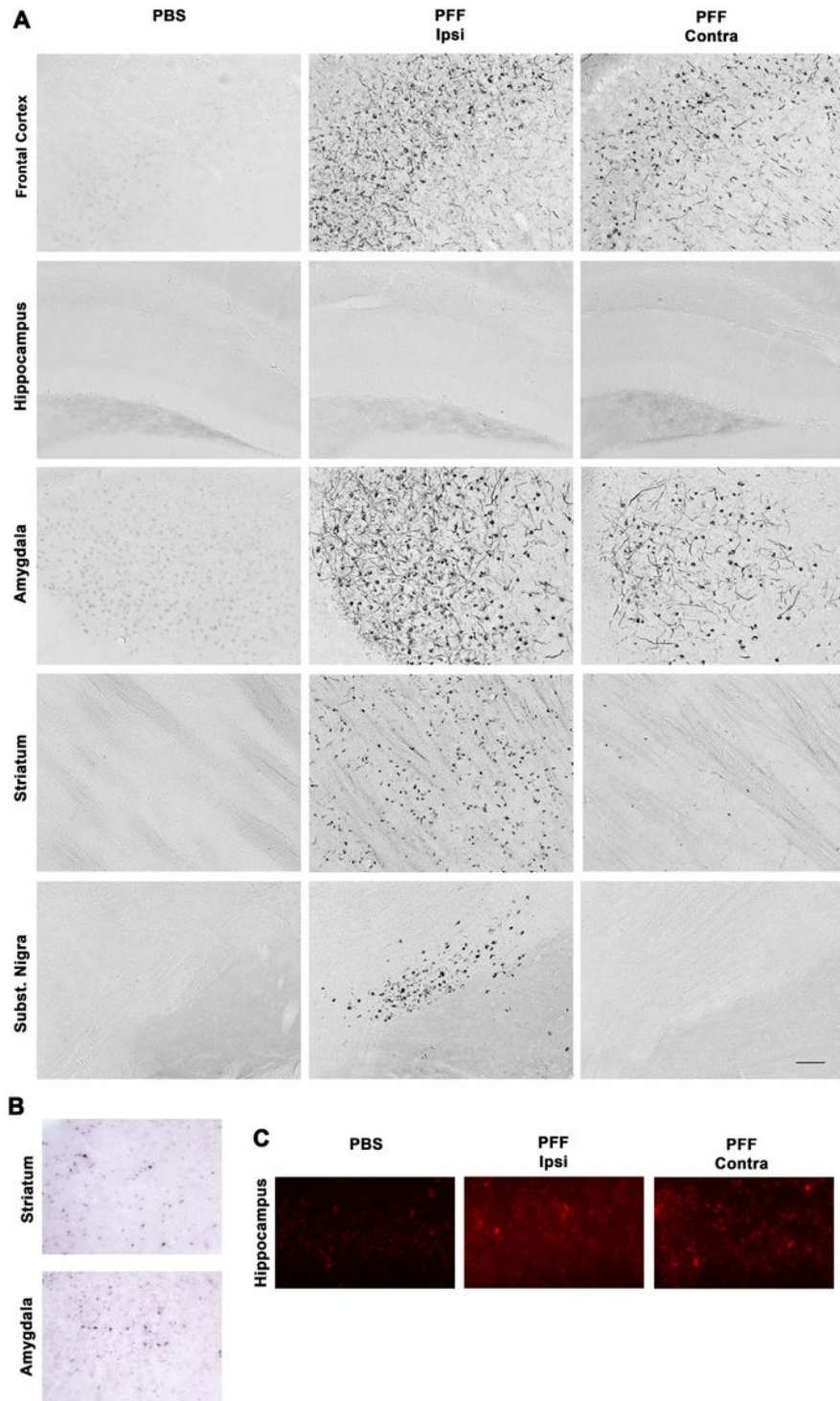


Figure 1

Intrastriatal injection of murine α -syn PFFs induced α -syn inclusions in various brain regions. Mice were euthanized 90 days after injection (90 dpi). A. PhosphoSER129 α -syn immunostaining showed numerous α -syn inclusions in neuritic and neuronal body structures in different brain regions. Widespread α -syn

inclusions were observed bilaterally in frontal cortex and the amygdala, ipsilaterally in the striatum and the Substantia Nigra (SN), and only minimally in the contralateral striatum and SN. None were observed in the hippocampus. No inclusions were observed in either side of the brains of PBS-injected control mice. Pictures show the ipsilateral side of these mice. B. Proteinase-K digestion on thin sections generated from paraffin-embedded tissue blocks revealed the presence of digestion-resistant α -syn inclusions stained for PhosphoSER129 α -syn. Shown here are ipsilateral striatum and amygdala for illustration. C. Proximity-ligation assay using a monoclonal PhosphoSER129 α -syn antibody showed the presence of enhanced levels of oligomeric forms of α -syn in the hippocampus of PFF-injected mice, where no inclusions could be detected 90 dpi, compared to PBS-injected controls. Scale bar = 250 μ m (A), 250 μ m (B), 25 μ m (C).

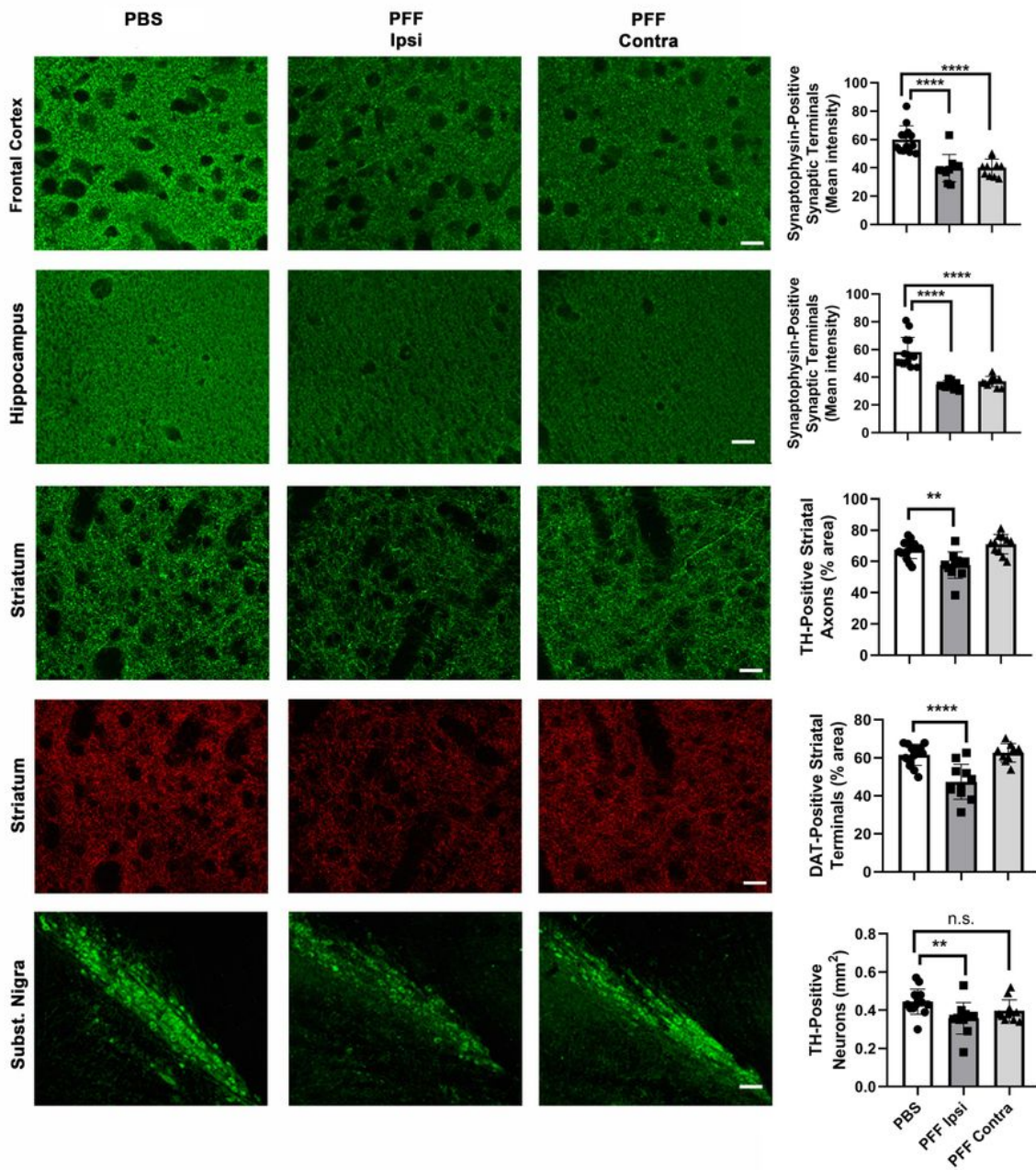


Figure 2

Intrastriatal injection of murine α -syn PFFs induced neurodegeneration in various brain regions. Mice were euthanized 90 dpi. In the frontal cortex and the hippocampus, a significant bilateral loss of synaptophysin-positive presynaptic terminals was observed (first two rows). In the striatum, a significant ipsilateral loss of TH-positive axonal fibers and DAT-positive synaptic terminals was observed (3rd and 4th row). In the SN, a significant loss of TH-positive neurons was observed only ipsilaterally. For group

comparisons and graphing, ipsilateral PBS measures were combined contralateral PBS measures, since they were similar. Pictures show the ipsilateral side of PBS-injected mice. **** $p < 0.0001$, ** $p < 0.01$, compared to PBS controls by Dunnett's post-hoc; $n = 6-11$ /group. Scale bars: 18 μm (for frontal cortical and hippocampal synaptophysin panels), 22.5 μm (for striatal TH and DAT panels), 80 μm (for Subst. Nigra panels)

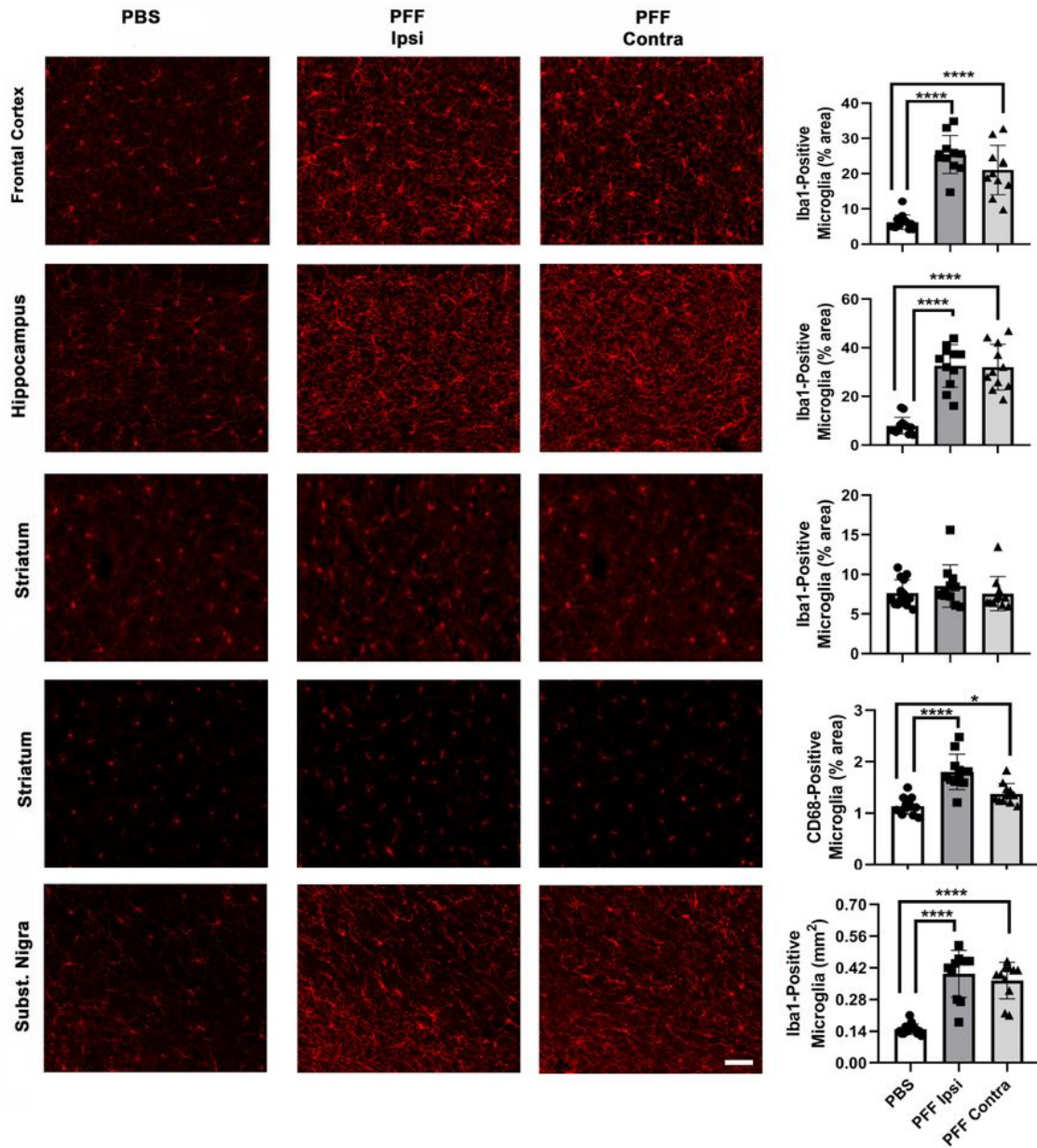


Figure 3

Intrastriatal injection of murine α -syn PFFs induced widespread microgliosis in different brain regions. Mice were euthanized 90 dpi. Panels show microgliosis measured on Iba1-stained sections of frontal cortex (upper row), hippocampus (second row), striatum (3rd row), and Subst. Nigra (last row), and on CD68-stained sections of striatum (4th row). A very strong microgliosis (up to 4x over control) was observed bilaterally in frontal cortex, hippocampus, and SN. No increase in Iba1 signal, but a significant bilateral increase in CD68 signal was observed in the striatum of PFF-injected mice. For group comparisons and graphing, ipsilateral PBS measures were combined contralateral PBS measures, since they were similar. Pictures show the ipsilateral side of PBS-injected mice. **** $p < 0.0001$, * $p < 0.05$, compared to PBS controls by Dunnett's post-hoc; $n = 6-11$ /group. Scale bars: 22.5 μ m (for all panels).

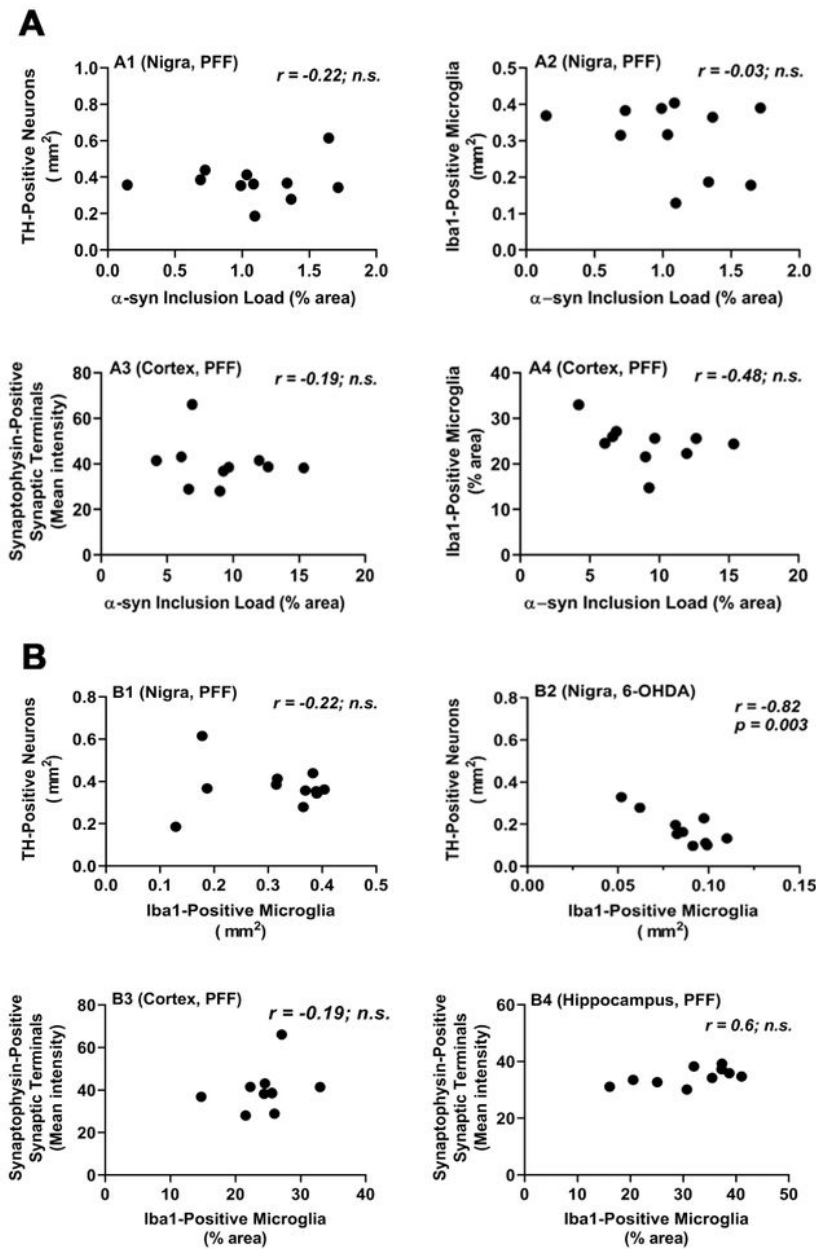


Figure 4

Different PD-related pathologies in the brains of mice injected intrastrially with α -syn PFFs do not correlate with each other. Mice were euthanized 90 dpi. A. α -syn inclusion load did not correlate with neurodegeneration (loss TH-positive neurons, A1) or with microgliosis (A2) in the SN (Nigra), nor with neurodegeneration (loss of synaptophysin-positive synaptic terminals, A3) or with microgliosis (A4) in the frontal cortex (Cortex). B. Microgliosis did not correlate with loss of TH-positive neurons in the SN after

intrastriatal PFF injection, but did so after intrastriatal injection of the toxin 6-OHDA. The microgliosis, measured on Iba1-stained section, was also much higher in the Subst. Nigra of PFF-injected mice than in that of 6-OHDA-injected mice. All measures shown are from the ipsilateral brain sides; similar observations were made for the contralateral sides of PFF-injected mice. Correlation analyses were done using Spearman rank test for data set including α -syn inclusion load measures, and with Pearson's test for data sets with the other measures.

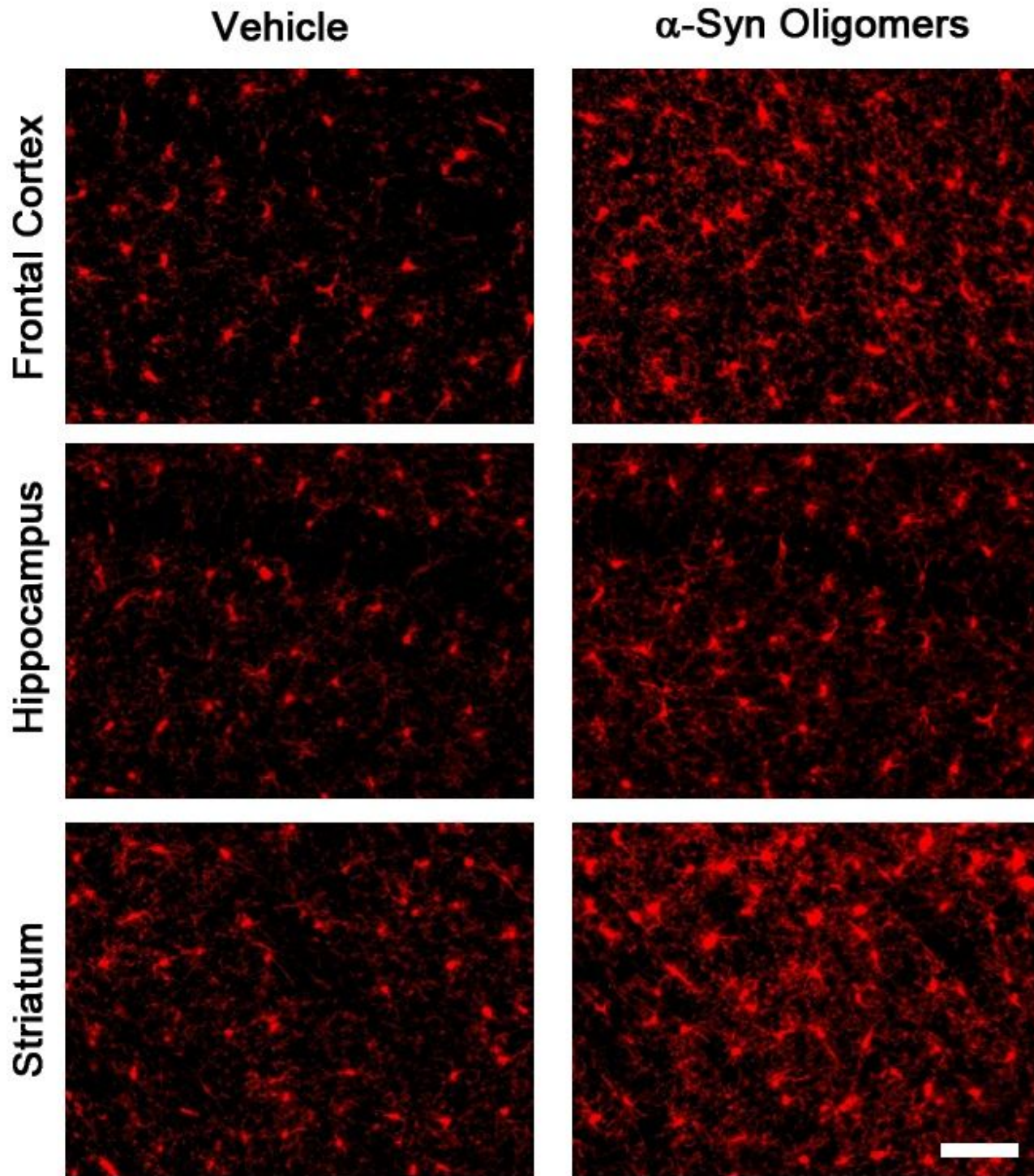


Figure 5

Strong microglial response after intrastriatal injection of α -syn oligomers. Oligomers were prepared and injections were performed as described in Materials and Methods. A strong microgliosis was observed in different brain regions 13 dpi, showing that α -syn oligomers are robust microglial activators in vivo. Scale bar = 40 μ m.

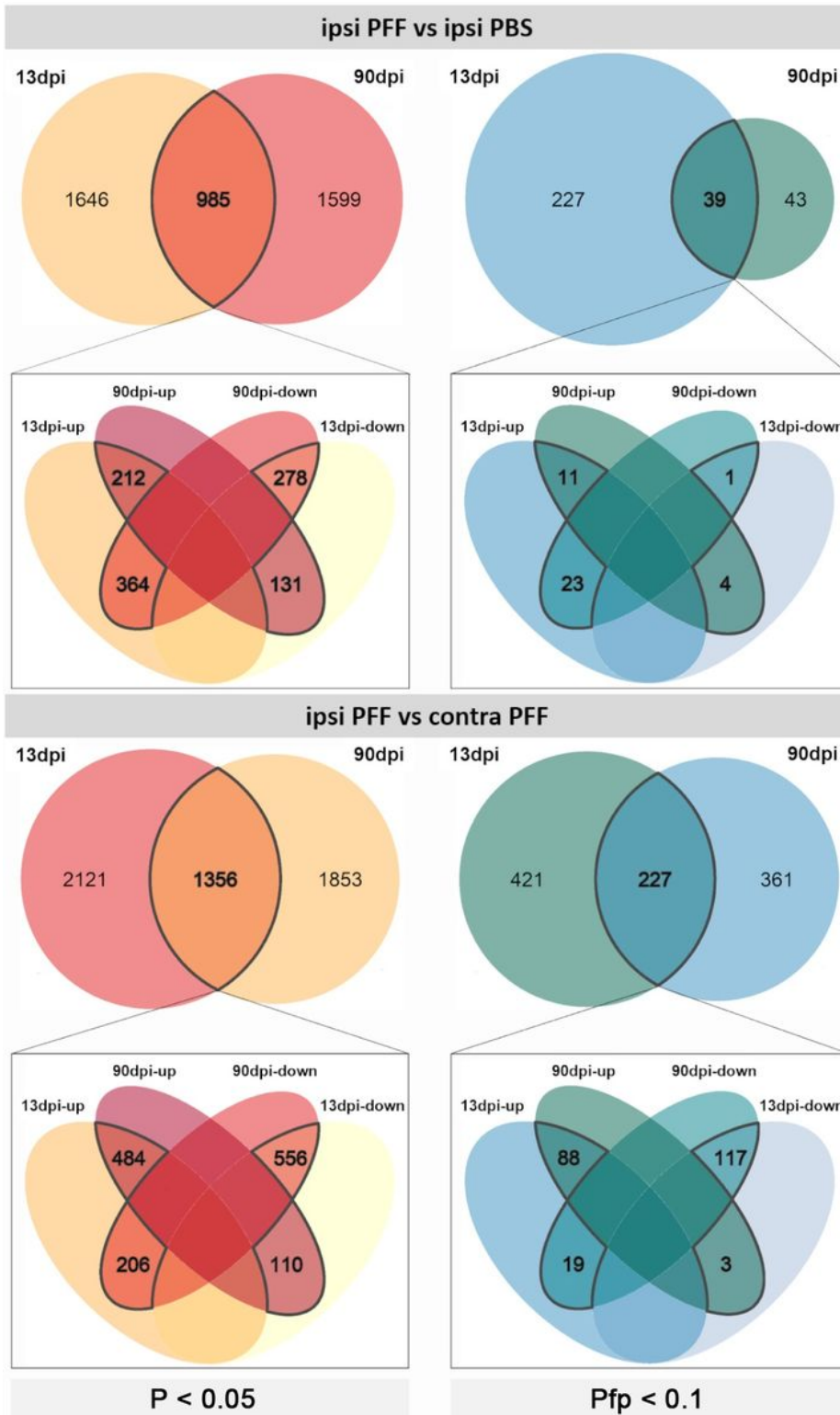


Figure 6

Differentially expressed genes induced in the ipsilateral ventral midbrain of mice at 13 dpi (A) and 90 dpi (B) after intrastriatal injections of α -syn PFFs. Comparisons for each time point were made between genes expressed in ipsilateral ventral midbrain of PFF-injected mice versus those in the ipsilateral ventral midbrain for PBS-injected mice (ipsi PFF vs. ipsi PBS, n = 6/group; upper 4 panels), and between genes expressed in ipsilateral ventral midbrain versus the contralateral ventral midbrains of PFF-injected mice (ipsi PFF vs. contra PFF, n = 6/group; lower 4 panels). Panels on the left show Venn diagrams with numbers of DEGs with a significance level of $p < 0.05$. Because some gene products have an effect within biological pathways while the expression of their genes may only change minimally, we show DEGs that emerge with this level of statistical stringency. In ipsi PFF vs. ipsi PBS, the number of DEGs was 2631 at 13 DPI, and 2584 at 90 DPI, with 985 DEGs common to both time points. In ipsi PFF vs. contra PFF, the number of DEGs was 3477 at 13 DPI, and 3209 at 90 DPI, with 1356 DEGs common to both time points. Panels on the right show Venn diagrams with the number of DEGs after adjusting for multiple hypothesis testing at $p < 0.1$. In ipsi PFF vs. ipsi PBS, the number of DEGs was 266 at 13 DPI, and 82 at 90 DPI, with 39 DEGs common to both time points. In ipsi PFF vs. contra PFF, the number of DEGs was 648 at 13 DPI, and 588 at 90 DPI, with 227 DEGs common to both time points. The Venn diagrams below the main ones indicate the number of DEGs that show enhanced ("up") versus decreased ("down"), as well as the overlaps, in the different comparisons.

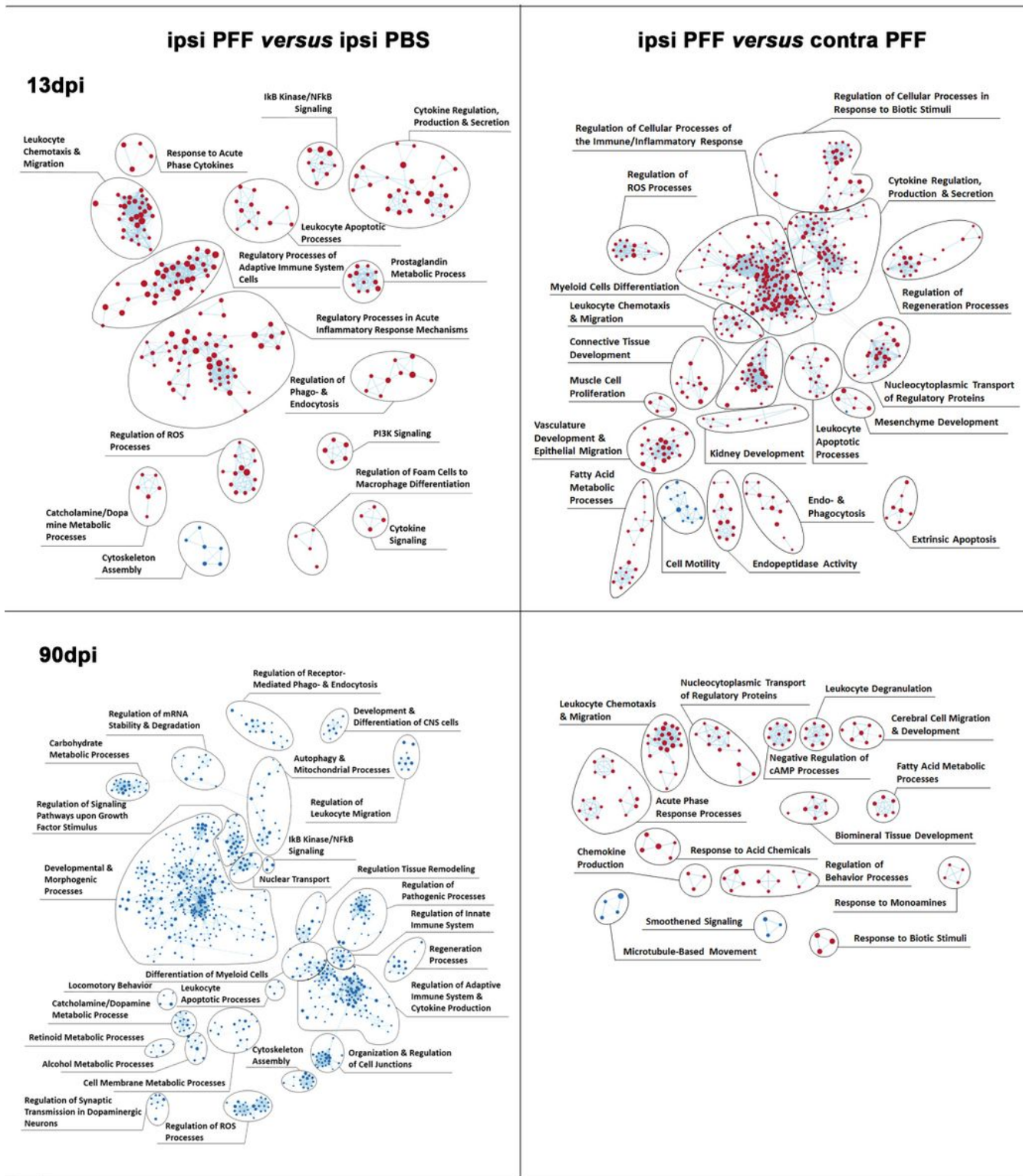
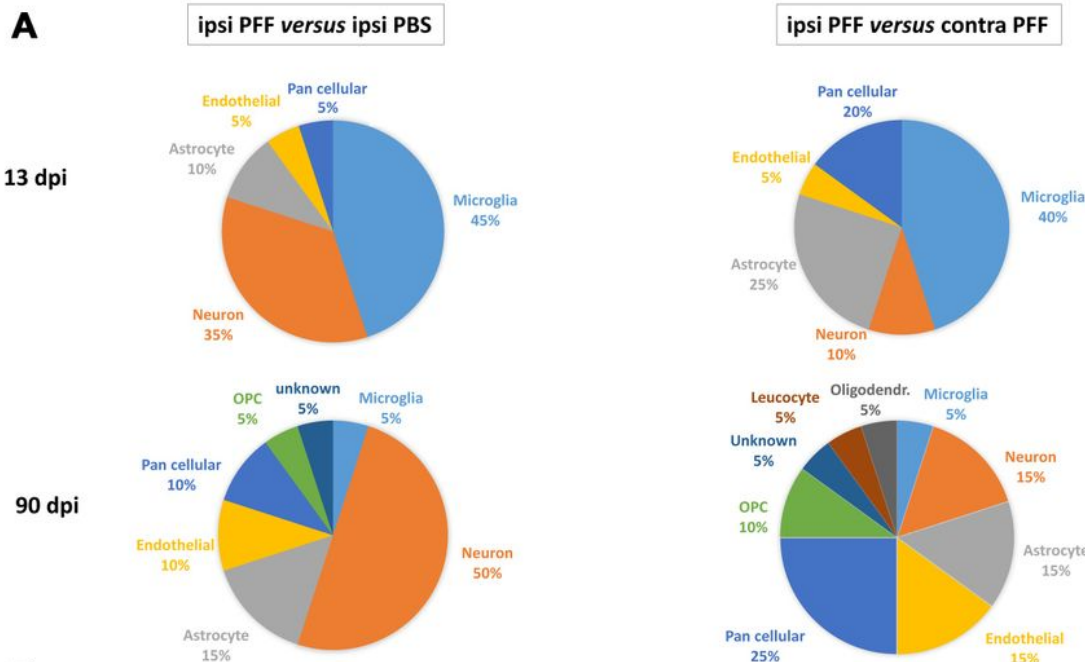


Figure 7

Enriched inflammatory pathways precedes neurodegeneration in mouse ventral midbrains after intrastriatal α -syn PFFs injection. Enrichment map of gene expression profiles were derived from GSEA. Statistics were done by weighted Kolmogorov-Smirnov, gene set size limits were set to min15 – max250. Details of curation procedure used to group BPs (represented as dots, either red if upregulated, or blue if downregulated) into high-level functional gene set clusters of BPs of related biological function

are described in Material & Methods. At 13 dpi, comparing ipsi PFF to either ipsi PBS or contra PFF, most BPs were upregulated and associated with gene sets related to immune and inflammation processes. This shows that, in the ipsilateral nigro-striatum, neuroinflammation precedes neurodegeneration (measurable at 90 dpi), and might contribute to its development. At 90 dpi, comparing ipsi PFF to ipsi PBS, all BPs, including those associated with inflammation gene sets, were downregulated, possibly reflecting the neurodegenerative process itself. Comparing ipsi PFF to contra PFF at this time point, most BPs were upregulated.



B

Gene Symbol	Protein Name	Cell type	FC	Pip	Gene Symbol	Protein Name	Cell type	FC	Pip
13dpi - ipsi PFF versus ipsi PBS					13dpi - ipsi PFF versus contra PFF				
<i>Slc6a3</i>	Sodium-dependent dopamine transporter	Neuron	2.600	2.94E-08	<i>Oxt</i>	Oxytocin	Pan cellular	-2.762	2.97E-13
<i>Oxt</i>	Oxytocin	Pan cellular	-1.519	1.64E-06	<i>Slc6a3</i>	Sodium-dependent dopamine transporter	Neuron	2.749	5.55E-10
<i>Lilrb4a</i>	Leukocyte immunoglobulin-like receptor, subfamily B, member 4A	Microglia	2.074	3.59E-06	<i>Tmem212</i>	Transmembrane protein 212	Astrocyte	-2.203	1.98E-09
<i>Spp1</i>	Osteopontin	Microglia	1.921	9.29E-06	<i>Stom3</i>	Stomatin-like protein 3	Pan cellular	-2.211	1.99E-09
<i>Cst7</i>	Cystatin F (leukocystatin)	Microglia	1.921	1.63E-05	<i>Lilrb4a</i>	Leukocyte immunoglobulin-like receptor, subfamily B, 4A	Microglia	2.437	2.18E-09
<i>Lpl</i>	Lipoprotein lipase	Microglia	1.795	1.70E-05	<i>Cst7</i>	Cystatin F (leukocystatin)	Microglia	2.412	3.46E-09
<i>Ccl3</i>	Chemokine (C-C motif) ligand 3	Microglia	1.819	1.72E-05	<i>Ogn</i>	Osteoglycin	Endothelial	2.296	4.44E-09
<i>Plin4</i>	Perilipin 4	Neuron	1.747	5.44E-05	<i>Clec7a</i>	C-type lectin domain family 7 member A	Microglia	2.348	8.01E-09
<i>Clec7a</i>	C-type lectin domain family 7 member A	Microglia	1.808	5.94E-05	<i>Ak7</i>	Adenylyl kinase 7	Astrocyte	-2.112	8.52E-09
<i>Ogn</i>	Osteoglycin	Endothelial	1.753	8.19E-05	<i>Spp1</i>	Osteopontin	Microglia	2.232	1.28E-08
<i>Avp</i>	Arginine vasopressin	Neuron	-1.149	1.30E-04	<i>Ccl3</i>	Chemokine (C-C motif) ligand 3	Microglia	2.367	1.33E-08
<i>Prg4</i>	Proteoglycan 4 (lubricin)	Astrocyte	1.777	1.72E-04	<i>Trh</i>	Thyrotropin releasing hormone	Astrocyte	-2.067	2.72E-08
<i>Gfap</i>	Glial fibrillary acidic protein	Astrocyte	1.633	1.85E-04	<i>Cdhr3</i>	Cadherin-related family member 3	Pan cellular	-1.996	5.01E-08
<i>Sim1</i>	Single-minded homolog 1	Neuron	-1.643	1.88E-04	<i>Slc17a7</i>	Vesicular glutamate transporter 1	Pan-cellular	2.288	1.10E-07
<i>Sgk1</i>	Serum/glucocorticoid regulated kinase 1	Microglia	1.675	2.00E-04	<i>Cd68</i>	CD68 antigen	Microglia	2.042	1.31E-07
<i>Cd68</i>	CD68 antigen	Microglia	1.683	2.07E-04	<i>Ccl6</i>	Chemokine (C-C motif) ligand 6	Microglia	1.980	1.96E-06
<i>Chrn6</i>	Neuronal acetylcholine receptor subunit alpha-6	Neuron	1.585	2.11E-04	<i>Prg4</i>	Proteoglycan 4 (lubricin)	Astrocyte	1.999	1.99E-06
<i>Zbtb16</i>	Zinc finger and BTB domain containing 16	Neuron	1.644	2.73E-04	<i>Dynlr2</i>	Dynein light chain roadblock-type 2	Astrocyte	-1.815	2.40E-06
<i>Tyrobp</i>	TYRO protein tyrosine kinase binding protein	Microglia	1.628	3.48E-04	<i>Fam183b</i>	Protein FAM183B	Neuron	-1.889	4.02E-06
<i>Fef1</i>	Fef family zinc finger protein 1	Neuron	-1.475	3.74E-04	<i>Ccl9</i>	Chemokine (C-C motif) ligand 9	Microglia	1.838	7.89E-06
90dpi - ipsi PFF versus ipsi PBS					90dpi - ipsi PFF versus contra PFF				
<i>Slc6a3</i>	Sodium-dependent dopamine transporter	Neuron	-2.383	1.51E-06	<i>Kcnj13</i>	Inward rectifier potassium channel 13	Pan cellular	2.556	7.66E-09
<i>Gh</i>	Growth hormone	Endothelial	2.007	1.09E-05	<i>Prg4</i>	Proteoglycan 4 (lubricin)	Astrocyte	2.593	1.03E-08
<i>Th</i>	Tyrosine hydroxylase	Neuron	-1.874	5.40E-04	<i>Ogn</i>	Osteoglycin	Endothelial	2.749	1.15E-08
<i>Slc18a2</i>	Synaptic vesicular amine transporter	Neuron	-1.782	1.33E-03	<i>Slc13a4</i>	Solute carrier family 13 (Sodium/sulfate symporter), member 4	OPC	2.330	4.82E-07
<i>Chrn3</i>	Neuronal acetylcholine receptor subunit beta-3	Neuron	-1.748	3.72E-03	<i>Ranbp3l</i>	RAN binding protein 3-like	Astrocyte	2.334	4.89E-07
<i>Slc5a7</i>	High affinity choline transporter 1	Neuron	1.630	3.95E-03	<i>Cym</i>	Ketimine reductase mu-crystallin	Pan cellular	2.193	1.06E-06
<i>Cyr61</i>	Cysteine-rich angiogenic inducer 61	Astrocyte	-1.692	4.44E-03	<i>Col6a1</i>	Collagen alpha-1(I) chain	Neuron	2.115	1.41E-06
<i>Gm10754</i>	Unnamed protein	Neuron	1.364	5.64E-03	<i>Rgs16</i>	Regulator of G-protein signaling 16	Endothelial	-2.062	1.61E-06
<i>Taf1d</i>	TATA box-binding protein-associated factor RNA polymerase I	Endothelial	1.560	7.33E-03	<i>Slit6</i>	SLIT and NTRK-like protein 6	OPC	-2.139	1.91E-06
<i>Chrn6</i>	Neuronal acetylcholine receptor subunit alpha-6	Neuron	-1.707	7.61E-03	<i>Stom3</i>	Stomatin-like protein 3	Pan cellular	-2.146	1.95E-06
<i>Kcnj13</i>	Inward rectifier potassium channel 13	Pan cellular	-1.556	1.38E-02	<i>C030013G03Rik</i>	Unknown	unknown	-2.018	2.12E-06
<i>Ret</i>	Proto-oncogene tyrosine-protein kinase receptor Ret	Neuron	-1.602	1.43E-02	<i>Car12</i>	Carbonic anhydrase 12	Neuron	2.151	2.28E-06
<i>Meis2</i>	Homeobox protein Meis2	Neuron	1.434	1.43E-02	<i>Pomc</i>	Pro-opiomelanocortin-alpha	Endothelial	-2.112	3.09E-06
<i>Slc13a4</i>	Solute carrier family 13 (Sodium/sulfate symporter), member 4	OPC	-1.358	1.56E-02	<i>Tcf7l2</i>	Transcription factor 7-like 2	Oligodendr.	-1.996	3.73E-06
<i>Ranbp3l</i>	RAN binding protein 3-like	Astrocyte	-1.288	2.13E-02	<i>Omd</i>	Osteomodulin	Astrocyte	2.155	4.48E-06
<i>Mir5098</i>	Stem-loop RNA, non coding	Pan cellular	1.507	2.13E-02	<i>Osr1</i>	Protein odd-skipped-related 1	Pan cellular	1.998	1.43E-05
<i>Aldh1a2</i>	Aldehyde dehydrogenase family 1, member A2	Neuron	-1.442	2.16E-02	<i>Nov</i>	Protein NOV homolog	Neuron	2.039	1.51E-05
<i>Trh</i>	Thyrotropin releasing hormone	Astrocyte	1.436	2.34E-02	<i>Trav7d-4</i>	T cell receptor alpha variable 7D-4	Leucocyte*	-1.783	1.69E-05
<i>C030013G03Rik</i>	Unknown	unknown	-1.540	2.36E-02	<i>Cox6a2</i>	Cytochrome c oxidase subunit 6A2, mitochondrial	Microglia	-1.837	1.73E-05
<i>Prkd</i>	Protein kinase C delta type	Microglia	-1.368	2.45E-02	<i>Oxt</i>	Oxytocin	Pan cellular	-1.835	1.85E-05

Figure 8

Top 20 DEGs in mouse ventral midbrain after intrastriatal injection of α -syn PFF indicate involvement of microglia in initial pathological events. Cellular source of DEGs was determined using the brain RNAseq database: <https://www.brainrnaseq.org/>. The top panels show pie charts with the cellular source of the 20 top DEGs when comparing ipsi PFF to ipsi PBS (left panels) or ipsi PFF with contra PFF (right panels) at 13 dpi and 90 dpi. At 13 dpi, comparing ipsi PFF with ipsi PBS or contra PFF, 45% and 40%, respectively, of the top 20 DEGs were microglial. At 90 dpi, comparing ipsi PFF with ipsi PBS, 50% of top 20 DEGs were neuronal, possibly a reflection of neurodegeneration. The bottom panel lists the gene products of the gene symbols, coded proteins, the associated cell type 20, the fold change (FC) and the pfp of all top 20 DEGs for each comparison.

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