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Spread of aggregates after olfactory bulb injection of α -synuclein fibrils is associated with early neuronal loss and is reduced long term

Corresponding author: Nolwen L. Rey. Van Andel Research institute, Center for Neurodegenerative Science. 333 Bostwick Avenue N.E., Grand Rapids, MI 49503, USA. Tel: +1-616-234-5828; Fax: +1-616-234-5129; Nolwen.Rey@vai.org.

Authors' contributions

N.L.R. designed the study, performed the injections, the immunohistochemistry, thioflavin S staining and the analysis, the interpretation of histological results, the quantitations and acquisition of brightfield images and some confocal images, and wrote the manuscript. S.G. performed the immunofluorescence and confocal microscopy, acquisition of brightfield images contributed to immunohistochemistry and edited the manuscript. J.A.S. provided critical expertise and wrote the manuscript. Z.M. performed the statistical analyses and provided critical expertise for heat map design. K.C.L., J.Q.T. and V.M.Y.L. synthesized and provided the PFFs for injections and edited the manuscript. P.B. contributed to the design of the study, interpretation of the results and wrote the manuscript. All authors gave input to the manuscript.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest

Dr. Brundin has received commercial support as a consultant from Renovo Neural, Inc., Roche, and Teva Inc, Lundbeck A/S, AbbVie, ClearView Healthcare, FCB Health, IOS Press Partners and Capital Technologies, Inc. Additionally he has received commercial support for grants/research from Renovo and Teva/Lundbeck. Dr. Brundin has ownership interests in Acousort AB and Parkcell AB. Dr. Trojanowski serves as an Associate Editor of *Alzheimer's & Dementia*. He may accrue revenue on patents submitted by the University of Pennsylvania wherein he is inventor including: Modified avidin-biotin technique, Method of stabilizing microtubules to treat Alzheimer's disease, Method of detecting abnormally phosphorylated tau, Method of screening for Alzheimer's disease or disease associated with the accumulation of paired helical filaments, Compositions and methods for producing and using homogeneous neuronal cell transplants, Rat comprising straight filaments in its brain, Compositions and methods for producing and using homogeneous neuronal cell transplants to treat neurodegenerative disorders and brain and spinal cord injuries, Diagnostic methods for Alzheimer's disease by detection of multiple MRNAs, Methods and compositions for determining lipid peroxidation levels in oxidant stress syndromes and diseases, Compositions and methods for producing and using homogenous neuronal cell transplants, Method of identifying, diagnosing and treating alpha-synuclein positive neurodegenerative disorders, Mutation-specific functional impairments in distinct tau isoforms of hereditary frontotemporal dementia and parkinsonism linked to chromosome-17: genotype predicts phenotype, Microtubule stabilizing therapies for neurodegenerative disorders, and Treatment of Alzheimer's and related diseases with an antibody. He is co-inventor on patents submitted the University of Pennsylvania wherein he is inventor that have generated income he has received from the sale of Avid to Eli Lilly including: Amyloid plaque aggregation inhibitors and diagnostic imaging agents.

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The authors declare no additional competing financial interests.

Ethics approval and consent to participate

The housing of the animals and all procedures were in accordance with the European international guidelines, with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health) and were approved by the Malmö-Lund Ethical Committee for Animal research (M109–13) and Van Andel Research Institute's IACUC (AUP 14-01-001). This article does not contain any studies with human participants performed by any of the authors (research on tissue derived from an autopsy is not considered as human subject research).

For the post-mortem human brain tissue, a consent for autopsy was obtained from the next-of-kin at the time of the death.

Nolwen L. Rey^{1,*}, Sonia George¹, Jennifer A. Steiner¹, Zachary Madaj², Kelvin C. Luk³, John Q. Trojanowski³, Virginia M.-Y. Lee³, and Patrik Brundin¹

¹Center for Neurodegenerative Science, Van Andel Research Institute, 333 Bostwick Avenue N.E., Grand Rapids, MI 49503, USA

²Bioinformatics and Biostatistics Core, Van Andel Research Institute, 333 Bostwick Avenue N.E., Grand Rapids, MI 49503, USA

³Department of Pathology and Laboratory Medicine, Institute on Aging and Center for Neurodegenerative Disease Research, 3rd Floor, Maloney Building, 3600 Spruce Street University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

Parkinson's disease is characterized by degeneration of substantia nigra dopamine neurons and by intraneuronal aggregates, primarily composed of misfolded α -synuclein. The α -synuclein aggregates in Parkinson's patients are suggested to first appear in the olfactory bulb and enteric nerves and then propagate, following a stereotypic pattern, via neural pathways to numerous regions across the brain. We recently demonstrated that after injection of either mouse or human α -synuclein fibrils into the olfactory bulb of wild type mice, α -synuclein fibrils recruited endogenous α -synuclein into pathological aggregates that spread transneuronally to over 40 other brain regions and subregions, over 12 months. We previously reported the progressive spreading of α -synuclein aggregates, between 1 and 12 months following α -synuclein fibril injections, and now report how far the pathology has spread 18 and 23 months post-injection in this model. Our data show that between 12 and 18 months, there is a further increase in the number of brain regions exhibiting pathology after human, and to a lesser extent mouse, α -synuclein fibril injections. At both 18 and 23 months after injection of mouse and human α -synuclein fibrils, we observed a reduction in the severity of α -synuclein aggregate pathology in some brain regions compared to others at 12 months. At 23 months, no additional brain regions exhibited α -synuclein aggregates compared to earlier time points. In addition, we also demonstrate that the induced α -synucleinopathy triggered significant early neuron loss in the anterior olfactory nucleus. By contrast, there was no loss of mitral neurons in the olfactory bulb, even at 18 months post-injection. We speculate that the lack of continued progression of α -synuclein pathology is due to compromise of the neural circuitry, consequential to neuron loss and possibly to the activation of proteolytic mechanisms in resilient neurons of wild-type mice that counterbalances the spread and seeding by degrading pathogenic α -synuclein.

Keywords

Parkinson's disease; alpha-synuclein; aggregates; spreading; neurodegeneration; propagation; olfactory bulb

Introduction

Parkinson's disease (PD) is characterized by the accumulation of intraneuronal inclusions of alpha-synuclein (α -syn) in the somata (Lewy bodies) or in neurites (Lewy neurites) of neurons [18]. α -Syn, as a presynaptic protein, is natively unfolded, but in Lewy pathology

α -syn misfolds into amyloid fibrils that form the hallmark inclusions of PD [11, 14]. Post-mortem studies of people with PD suggest that α -syn pathology progresses in the brain in a stereotypical pattern over decades, first starting in the olfactory bulb (OB) and the dorsal motor nucleus of the vagus, and then spreading to central brain regions, ultimately reaching cortical areas [6–8, 13]. Observations also suggest that the propagation is occurring along neural connections, since the regions that are sequentially involved are also anatomically interconnected [13].

Severe α -syn pathology appears several years prior to the diagnosis of the classical motor symptoms of PD, and is accompanied by non-motor deficits such as olfactory dysfunction, constipation and sleep disturbances [52]. One of the hallmarks of PD is severe neuronal loss in the substantia nigra that leads to the loss of dopaminergic signalling and the appearance of motor symptoms. Neuronal loss is also observed in other brain regions in PD, such as the anterior olfactory nucleus (AON) [50], the locus coeruleus (LC) [17] and the amygdala [22]. In addition, the volumes of the piriform cortex (PC), the amygdala, the OB and the orbitofrontal cortex are reduced [33, 50, 67, 74] (reviewed in [60]). The neuropathology in all of these brain regions likely contributes to the non-motor symptoms of PD [24, 54]. Based on the ability of misfolded α -syn to aggregate and further recruit endogenous α -syn, the α -syn transmission hypothesis posits that misfolded α -syn can form proteopathic seeds that template normal α -syn to misfold, undergo intra-axonal transport to other brain regions and propagate synucleinopathies through iterative repetition of this process, perpetuating a vicious cycle [16, 20, 31, 40, 46]. This process is suggested to underlie the spatio-temporal progression of α -syn pathology observed in PD brains. Several laboratories have developed models of pathology induction and spreading by injecting recombinant fibrillar α -syn, or brain extracts from PD patients, into the brain, muscles, peritoneal cavity or circulatory system of α -syn-overexpressing or wild type rodents [2, 12, 36–39, 41, 49, 51, 56, 59, 61, 63]. These injections induce α -syn aggregate pathology in neuronal populations proximal to the injection site, and in more distant regions with neuronal connections to the site of injection. We recently demonstrated that pathology induced by unilateral injection of human or mouse α -syn preformed fibrils (PFFs), designated huPFFs or mPFFs, respectively, into the OB of 3 month old wild type (WT) mice can propagate sequentially over multiple synaptic relays, reaching numerous ipsi- and contralateral brain regions after 12 months, including brain stem areas (e.g. a few aggregates in the substantia nigra, LC and raphe nucleus). The spreading of α -syn aggregates was coupled to progressive deficits in olfaction [59]. Taken together, the model involving huPFFs and mPFFs injections into the OB of WT mice results in the spread of pathology and olfactory deficits that mimic prodromal PD within 1 to 12 months post-injection [59]. In the present study, we further define the consequences of these huPFFs and mPFFs injections into the OB and determine, first, the extent of α -syn pathology at 18 and 23 months after injection and second, if there is cell loss in the olfactory bulb and an additional olfactory region (AON).

Material and methods

Study Design

The goal of this study was to establish if injection of PFFs into the OB of mice leads to neurodegeneration and what the pattern of α -syn pathology is 18–23 months post PFF injection. All experiments were performed blinded. After surgery, animals were assigned a name independent of the experimental group they were part of. That name was used for assessing pathology location and scoring. For other subsequent analysis, a second individual assigned new names to stained slides so the experimenter would conduct the analysis blinded to the identity of the mice. Further experimental detail, protocols, including animal care/handling and number of biological/technical replicates, are presented in the following sections and in the figure legends.

Animals

We used 48 C57Bl/6J female mice which were 3 months old at the start of the study, purchased from Charles River Laboratories (Sweden) and the Jackson Laboratory (USA). The mice had constant access to food and water, and were housed five to six per cage under a 12-h light/12-h dark.

Stereotactic injections

We prepared pre-formed fibrils (PFFs) from recombinant full-length mouse α -syn and full-length human α -syn. Purification and assembly of the proteins was performed as described [73]. PFFs and soluble mouse α -syn were stored at -80°C . On the day of the surgery, PFFs were thawed, sonicated as described by us previously [59], and kept at RT during the surgical procedure. Recombinant soluble mouse α -syn used for controls was kept on ice during the surgical procedures.

Forty-two wild-type mice received stereotactic injections of sterile phosphate buffered saline (PBS), mouse wild-type α -syn monomers (mMs), mouse wild-type α -syn PFFs (mPFFs) or human wild-type α -syn PFFs (huPFFs) into the OB unilaterally, following our previously published protocol [58, 59]. Six additional mice did not undergo any surgical procedures (non-injected group, aged-matched).

Some of the mice described in this study, which were left to live 1–12 months after surgery, are part of an experiment that we have reported on previously [59]. An additional 42 mice that were not euthanized prior to compiling our previous report were aged to a maximum of 23 months post-injection. For our current analyses, we used the following mice that were not part of our previous publication [59]: 9 months, 18–19 months (called “18 months” in the rest of the article) and 22–23 months (called “23 months” in the rest of the article) post-injection delays. In addition, we reexamined mice from the 1, 3, 6 and 12-months old groups from our previous work [59], for additional analyses (see Online Resource 1 for clarification). Only groups with 3 animals or more were used for analyses.

Preparation of the tissue

Mice were perfused transcardially with 0.9% saline followed by 4% PFA, brains were collected and post-fixed as described previously [58, 59]. We stored the brains at 4°C until sectioning. The entire brain of each mouse was sectioned using a freezing microtome into 30 µm free-floating coronal sections, and stored in antifreeze solution at 4°C until immunostaining.

Sections from human brain with tau pathology (Alzheimer's disease; angular gyrus) and sections from human brain with TDP-43 pathology (FTLD-TDP, cingulate cortex) were obtained from the Center for Neurodegenerative Disease Research, University of Pennsylvania, Philadelphia. These post-mortem brains came from longitudinally followed human subjects who were assessed neuropathologically as described [71]. Prior to 6 µm-thick sectioning, the samples were fixed in 10% neutral-buffered formalin, and paraffin embedded, and stored at RT.

Immunohistochemistry

Paraffin embedded tissue was deparaffinised in xylene and rehydrated. For 1D3 and AT8 detection, heat-induced antigen retrieval was performed (citrate buffer, pH 6) prior to staining mouse and human samples. We stained coronal free-floating sections (mouse tissue) and sections on slides (human brain samples) using primary antibodies against phosphorylated α-syn (Ser129) (pser129, rabbit, 1:10000, Abcam, Ab51253), hyperphosphorylated TDP-43 (pS409/410) (TAR5P-1D3, rat monoclonal, 1:200, Ascension, Munich, Germany) [45], or hyperphosphorylated tau (pS202/T205) (AT8, mouse monoclonal, 1:10000, Thermo Scientific). Secondary biotinylated anti-rabbit serum (goat, 1:500, Vector Laboratories, BA-1000), anti-rat (goat, 1:500, Vector Labs, BA-9400) or anti-mouse (goat, 1:500, Vector labs, BA-9200) were used. We used a standard peroxidase-based method with DAB to detect the antibody (Vectastain ABC kit and DAB kit, Vector Laboratories). Slides were dehydrated and coverslipped with Cytoseal 60 mounting medium (Thermo Scientific) and analysed by conventional light microscopy on a NIKON Eclipse Ni-U microscope (Nikon); we captured images with a Retiga 2000R digital camera using NIS Elements AR 4.00.08 software (Nikon).

For analysis of pser129 pathology spreading in the brains of mice, we stained with the anti-pser129 serum a whole series of coronal sections (210 µm intervals between consecutive sections) from 3–5 animals per group (non-injected, PBS-, mMs-, mPFFs-, huPFFs-injected groups). We assessed in a blinded manner the presence of pser129-positive inclusions by screening every section at 20x magnification using a NIKON Eclipse Ni-U microscope.

Immunofluorescence staining

We stained coronal free-floating sections with primary antibodies against pser129 (pser129, rabbit, 1:5000; Abcam, Ab51253) and NeuN (mouse, 1:1000, Millipore, Mab377), and secondary antibodies AlexaFluor 488 anti-mouse (goat, 1:500, Jackson ImmunoResearch Laboratories, 115-545-146) and AlexaFluor 594 goat anti-rabbit (goat, 1:500, Jackson ImmunoResearch Laboratories, 111-585-144). The staining with thioflavin S was performed in accordance with our previous work [59].

Stained slides were blind coded before analysis and were imaged with an inverted confocal laser microscope Nikon Eclipse Ti-E. Thioflavin S staining was imaged by averaging 4x scans for each z step of the stack to remove noise using NIS Elements AR 4.00.08 software (Nikon). For all other fluorescence staining, we acquired images by single scan per stack-step. We post-treated the confocal stacks with a kernel-3 median filter to remove noise and generated all final images using NIS Elements AR 4.00.08 software (Nikon).

Scoring of pser129 pathology and heat map

Neuropathology detected by antisera to pser129 was analysed on blind coded slides. We assessed the presence of pser129 inclusions on a whole series of coronal sections (210 μ m intervals between sections) from 3–5 animals per experimental group, employing a simplified version of the method used previously [59]. We screened every single section at 20x magnification using a NIKON Eclipse Ni-U microscope and we assigned a score to each brain region, from 0 to 4, based on the relative abundance of pser129-positive inclusions (cell bodies and neurites) (0 = no aggregates, 1 = sparse, 2 = mild, 3 = dense, 4 = very dense) Brains regions in the diagrams presented on Figure 1b–d were colour-coded according to the score from 0 to 4 that represents of the median of animals per group.

To generate a heat map (Fig 3a–c), we calculated the average score values per group per brain region [27], and represented the data on a heat map programmed with the software R [53]. Representative images of pser129 staining presented in Fig 1a were acquired at 40x or 60x magnification on the same microscope.

Densitometry of pser129 pathology measured by ImageJ

We assessed pser129 pathology density in the OB, the AON, the anterior and posterior PC and the Ent in mM^s-, huPFFs, mPFFs-injected mice 12 and 18 months post-injection, and in non-injected age-matched animals. 20x magnification images were acquired using a NIKON Eclipse Ni-U microscope (Nikon), using the same exposure time for all the images. OB images were acquired using the condenser, while images of the AON, PC and Ent were acquired without the condenser lens (to take into account a thicker layer of the section). In the OB, images were acquired on 3 sections separated by 420 μ m intervals (between Bregma 4.9 mm and 4 mm), the last section being the most posterior section before the appearance of the AOB (Bregma 4 mm approximately). Three images of the medio-lateral AON were acquired (420 μ m intervals between sections, covering the whole AON; between Bregma 3 mm and 2 mm). For the Ent, 3 images were acquired (840 μ m intervals between sections imaged, between Bregma –2.90 mm and –4.40 mm). We analysed separately the anterior and the posterior PC since they receive inputs from different brain regions (for review, [5]). We acquired 4 pictures of the anterior PC (630 μ m intervals between sections imaged, localized between Bregma 2.34 mm and 0.2 mm); and 4 pictures of the posterior PC (630 μ m intervals between sections imaged, localized between Bregma 2.4 mm and 0.34 mm). Images were then processed in ImageJ64 [55] as described in Online Resource 2, to create a mask (to exclude the background) that redirects to the original image for analysis. For brain regions like the PC that might not fit entirely in the field, we drew the contour of the region, and the analysis was performed within the contoured area (Online Resource 2b), we measured the area and the mean grey value of the area that was positively stained. We then

calculated the grey value per square pixels per image ($\text{A.U.}/\text{px}^2 = \text{mean grey value} \times \text{area stained}/\text{total area assessed}$). We then calculated the average grey value per square pixels for each brain region of each animal ($n=3-5/\text{group}$, details in the legend of Fig 4). We then calculated the average grey value per square pixels for each group and region of interest. Individual data were then analysed using linear mixed-effects models with a random intercept for each sample via the 'lme4' (<http://lme4.r-forge.r-project.org/>) package in R v3.2.1 (<https://cran.r-project.org/>). Linear contrasts with false discovery rate adjustments were used to test specific hypotheses and account for multiple testing (brain region, time point, experimental group, ipsi- versus contralateral region). The inclusion of the random intercepts accounts for the auto-correlation in these data and ultimately yields better model performance and increased accuracy [30]. Normality and homoscedasticity assumptions were verified using quantile-quantile plots and scattered plots of the residuals. Outcomes were natural log transformed, which improved modelling assumptions. For ease of interpretation, effects were back-transformed to estimate fold change and the 95% confidence interval. Graphs were prepared using Prism 6.0, GraphPad.

Mitral cell density

We measured the density of mitral cells in the OB on cresyl violet stained sections. The staining and the analysis were performed as described previously [59]. Homoscedasticity was verified with a Brown-Forsythe test. We then performed a two-way ANOVA with repeated measures using Prism 6.0, GraphPad. As no significant effect of PFFs injection was observed, we did not perform any post-hoc tests.

Cresyl violet-positive cell counting by stereology in the AON

Cresyl violet stains all cells including large neurons, small neurons and glial cells that cannot be distinguished by morphology from the small neurons (Fig 5a, arrows) [29]. The AON contains small-sized interneurons [3] (subpopulation of GABAergic neurons [28]), which are similarly sized to glial cells. The glial population have been estimated to account for 10–36% of the total cell population in the mouse brain [23, 44]. We quantified cresyl violet-positive cells by stereology in the ipsilateral and contralateral AONs of mMs-, mPFFs, huPFFs-injected mice 6 and 18 months post-injection, and non-injected age-matched controls. Large neurons with faint cresyl violet-positive signal (indicated by arrowheads on micrographs in Fig 5a) and small dark stained cells (indicated by arrows on micrographs in Fig 5a) were quantified separately. Hence, our quantifications of the dark stained cells likely also include glial cells that account for only a small proportion of cells present in the AON, but the large faint cresyl violet-positive cells includes only neurons. We used a computer-assisted mapping and cell quantification program (Stereo Investigator, MBF Bioscience, Williston, USA) coupled to a Zeiss Imager M2 microscope (Zeiss, Thornwood, NY). We analysed a full series of sections (5–7 sections) per animal spaced by $210 \mu\text{m}$ (section interval = 8), in 3–5 animals per group. Contours of the region were drawn at 2.5x magnification. Quantifications were performed at 100x (oil objective) using a counting frame of $40 \mu\text{m} \times 40 \mu\text{m}$, grid size was set to 100 (medial-lateral) \times 140 (dorsal-ventral) μm , with a guard zone of $3 \mu\text{m}$, and dissector height set at $8 \mu\text{m}$. These parameters used for AON stereology analyses were set to assess at least 300 total cells per animal and side, and have an error coefficient ($m=1$) below 0.09. We then calculated the average cell number per group

and region of interest and analysed the data using negative binomial mixed-effects models with random intercepts for each individual, using the 'lme4 package (<http://lme4.r-forge.r-project.org/>) in R v3.2.1 (<https://cran.r-project.org/>). A negative binomial regression was chosen because these are count data and the mixed-effects allow higher performance of the model and increased accuracy [30]. Linear contrasts with false discovery rate corrections were used to adjust for multiple testing (cell morphology, time point, experimental group, ipsi- versus contralateral AON). Normality and homoscedasticity assumptions were verified using quantile-quantile plots and scatter plots of the residuals. Graphs were prepared using Prism 6.0, GraphPad.

Results

We hypothesised that α -synucleinopathy would continue to spread at survival times exceeding one year post-injection, i.e. beyond the brain regions previously reported to be affected 12 months after such injections [59]. We previously reported that after 3 months following mPFFs injection, severe and widespread α -syn pathology in many regions directly or indirectly connected to the injection site was observed [59]. After 12 months, α -syn pathology induced by mPFFs reached more than 40 different brain sub-regions, including contralateral structures [59]. In the current study, we also hypothesise that following injection of either huPFFs or mPFFs α -syn into the OB of wild-type mice, α -syn aggregation in olfactory pathways would lead to neuron loss, which could contribute to the olfactory deficits previously described [59]. In our earlier study, we demonstrated that there is no loss of mitral cells in the OB at 6 months post-injection [59].

Absence of pser129-unspecific staining in white matter tracts

As in our previous work [59], we detected pathology using an antibody against pser129 α -syn. This post-translational modification of α -syn is indicative of abnormal α -syn in synucleinopathies [15, 48] and is a reliable marker for α -syn inclusions [4, 57]. Concerns have been raised previously about pser129 antibodies and their possible binding to phosphorylated neurofilament subunit L in white matter tracts [64]. Therefore, we tested our pser129 staining protocol in white matter and found that it results in no detectable staining in different white matter tracts of non-injected animals and mMs injected animals (Online Resource 3a).

Widespread mild α -syn pathology is present at 18 and 23 months post-injection

When assessing pathology in the whole brain of PFFs-injected mice, we observed widespread pser129-positive pathology in neurites and cell bodies including ipsilateral and contralateral olfactory regions, and non-olfactory regions (Fig 1a). We analysed at least one series of sections from the entire brain of all groups of mice, i.e. PBS-, mMs-, huPFFs, mPFFs-injected and non-injected age-matched mice. Consistent with the strategy used to assess α -syn pathology in publications on human synucleinopathy brains [4, 6, 8], and our own prior work [59], we considered the presence of a single pser129-positive cell or neurite to indicate that pathology was present.

α -Syn pathology was present in numerous brain regions 18 months after mPFFs and huPFFs injection and 23 months after huPFFs injections (Fig 1a), including in olfactory regions and non-olfactory regions that are directly and indirectly or transneuronally connected to the injected OB. We scored the severity of α -syn pathology in the entire mouse brain [59]. At 18 and 23 months post-injection, the α -syn pathology in mice injected with PFFs was mild to moderate (Fig 1b–d). In addition, we assessed several control groups (mMs, PBS-injected mice, and non-injected age-matched mice). PBS-injected mice and non-injected mice did not display signs of α -syn pathology at 18- and 23-months post-injection time points (data not shown, n=3–4 per group). Two out of 4 mice injected with mMs assessed at 18 months post-injection, and one mouse out of 4 assessed at 23 months post-mMs-injection exhibited a very low number of pser129-positive neurites in the ipsilateral OB, piriform cortex (PC) and central amygdala (CeL) (Fig 1a).

Pser129 inclusions at 18 months are present within neurons, and are positive for Thioflavin S

Next, we used confocal microscopy to determine whether pser129-positive inclusions are present within the neurons of the AON. As expected, and consistent with our previous report [59], NeuN-positive cells in the AON displayed pser129-positive inclusions (Fig 2a). We also observed pser129-positive inclusions in non-NeuN positive cells (data not shown).

To confirm that the inclusions observed with pser129 antibody in our model are mature amyloid inclusions, we performed a staining with thioflavin S (Fig 2b). Inclusions in PFFs injected mice 18 and 23 months post injection are thioflavin S-positive, indicating mature amyloid structures.

PFFs-induced pathology does not trigger the aggregation of tau and TDP-43

Since TDP-43 and tau pathology co-occur with α -syn pathology in PD, PDD and DLB in humans [43, 69], and the presence of tau pathology influences α -syn pathology load in PDD [26, 70], we investigated whether PFFs injections could trigger tau and TDP-43 pathology after long post-injection times. We assessed tau and TDP-43 pathology in mice injected with PBS, huPFFs and mPFFs 18 months post-injection. Tau and TDP-43 pathologies were detected by immunohistochemistry against hyperphosphorylated tau (AT8), and against hyperphosphorylated TDP-43 (1D3) respectively, as commonly used [19, 45]. Sections from post-mortem tissue of a person with Alzheimer's disease (AD) with abundant neurofibrillary tangles and of a person with frontotemporal lobar degeneration with severe TDP-43 pathology were used as positive controls for the staining procedure. While tau and TDP-43 pathology were evident in human tissue, no pathology could be detected in PFFs-injected mice (Online Resource 3b), indicating that PFFs injections did not trigger tau and TDP-43 pathology 18 months post-injection.

Changes in α -syn pathology across time

To investigate the kinetics of α -syn pathology propagation after long delays post-injection, we calculated the mean score of pathology (scoring in all the brain regions where pathology could be detected at any time between 1 and 23 months post-injection). Data are summarised in heatmaps (Fig 3) and include 18 and 23 month-old animals presented in this study,

animals assessed at 1, 3, 6 and 12 months old that were described in our previous article [59], and an additional time point, at 9 months post-injection. Heatmaps are based on the same scoring method as the diagrams (Fig 1b–d) but provide a better overview of the data.

No pathology was detectable at 1, 3, 6, and 12 months post-injection in mMs-injected mice (score 0) (Fig 1a, Rey et al. 2016). As expected, no pathology was detected at 9 months post-injection (Fig 1a), but mild pathology (mainly in neurites, occasionally in the cell body) appeared in olfactory regions in mice injected with mMs at 18 months post-injection, and in additional non-olfactory regions at 23 months (Fig 3a) (score between 0 and 1).

mPFFs injection—Mice injected with mPFFs progressively developed severe and widespread pathology between 1 and 12 months post-injection (Fig 3b, [59]), exhibiting pathology scores between 2 and 4 in many brain regions after 6-months delay. At 18 months post-mPFFs injection we observed α -syn pathology in brain regions that did not contain α -syn pathology at 12 months (Fig 1a, 1b, Fig 3b, [59]) (i.e. ipsilateral peduncular cortex (DP), retromammillary nucleus (RML), ipsilateral and contralateral infra-limbic cortex (IL), contralateral OB and contralateral anterior part of the paraventricular thalamic nucleus (PVA), indicating a progressive spread of α -syn pathology. By contrast, some regions that exhibited α -syn pathology at 12 months post-injection (Fig 3b, [59]) did not display pser129 inclusions at 18 months post-injection, particularly some that are indirectly connected to the injection site (Fig 3b) (e.g. ipsilateral Th; association area of the temporal cortex, TeA; Substantia nigra, SN; Locus coeruleus, LC; contralateral entorhinal, Ent, and ectorhinal, Ect, cortex). An apparent decrease in the pathology scores after 18 months suggest a reduction in the density of pathology in specific brain regions compared to the 12 months delay.

HuPFFs injection—Next, we assessed α -syn pathology following the injection of huPFFs into the OB. Between 1 and 12 months post-injection, the pathology score remained mild (between 0 and 2) in most of the brain regions affected, except the ipsilateral AON and the PC, with scores up to 4 (Fig 3c). α -Syn pathology after 18 months emerged in many previously spared brain regions (e.g. ipsilateral and contralateral insular cortex (Ins), ipsilateral primary and secondary sensory and motor cortical areas (S1, S2, M1, M2 respectively), SN, raphe nucleus (RN), LC, iDP, IL, RML; and contralateral central nuclei of the amygdala (CeL, CeM, CeA), hippocampus (molecular layer, dentate gyrus and Cornu Ammonis 2), lateral hypothalamic area (LH) and peduncular part of the lateral hypothalamic area (PLH) and olfactory tubercle (OT) (Fig 3c). At 23 months post-injection, pathology was still present in numerous brain regions, but no longer observed in several previously affected brain regions (e.g. ipsilateral M1; M2; S1; S2; TeA; SN; caudate putamen, CPu; anterior and posterior basomedial nuclei of the amygdala, BMA, BMP; contralateral Ins; anterior, ventral and posterior basolateral amygdala, BLA, BLV, BLP) (Fig 3d), which may indicate a regression of pathology at extended periods after PFFs injection. In analogy to our findings after mPFFs injections, huPFFs injected mice displayed mild to moderately severe pathology at the 18 and 23 month time points (Fig 3b–c). The present results suggest that the amount of α -syn pathology induced by huPFFs in a given brain region does not increase further beyond what is seen one year after injection, despite a greater number of brain regions being affected at 18 months.

α -Syn pathology density

To further investigate the changes in α -syn pathology across time and the potency of the different PFFs to induce pathology, we measured the density of pser129-positive inclusions 12 and 18 months post mMs, huPFFs and mPFFs injection in the OB, the AON, the PC and the Ent (Fig 4) (methods summarized in Online Resource 2, statistical analyses are detailed in Online Resource 5).

First, as expected, the density of pathology was significantly higher in the ipsilateral side than the contralateral side after injection of huPFFs and mPFFs (Fig 4, Online resource 5a). In mMs-injected group, pathology is very sparse or absent. HuPFFs induced significantly more pathology than mMs, while mPFFs induced up to 10 times more abundant pathology than elicited by huPFFs at 12 months post-injection (Fig 4, Online resource 5b), indicating that mPFFs were more potent than huPFFs.

The pathology induced by mMs increases significantly between 12 and 18 months in the AON and the PC while pser129 burden did not change between 12 and 18 months after huPFFs-injection (Fig 4, Online resource 5c). In contrast, mPFFs-induced pathology decreased significantly at 18 months in the ipsilateral OB (Fig 4a–b, Online resource 5c), in the ipsilateral and contralateral anterior PC (Fig 4e–f, Online resource 5c), posterior PC (Fig 4g–h, Online resource 5c) and Ent (Fig 4i–j, Online resource 5c), reaching similar levels as huPFFs (AON, aPC, pPC, Ent).

We also compared the density of pathology between the anterior and posterior PC, and no differences were observed (Online resource 5d). Altogether, our results confirm the decrease of pathology density after 18 months post mPFFs injections, and demonstrate that there is no further increase in the density of α -syn pathology induced by huPFFs injection past 12 months post-injection, despite the involvement of additional brain regions (Fig 3).

Severe cell loss in the AON but not in the OB

To explore if neural cell death contributes to the olfaction deficits we previously reported [59], we assessed cell loss by stereology at 6 and 18 months post-injection in the OB and in the AON. The AON olfactory region receives direct projections from the OB and coordinates the activity of the olfactory system between both hemispheres [9].

In the ipsilateral AON, the total number of cresyl-positive cells 6 months post-injection decreased by 55–58% after huPFFs injection and by 39–42% after mPFFs injection (Fig 5a–b, Online resource 6b). In the contralateral AON, the number of cresyl violet-stained cells 6 months post-injection decreased by 61–62% in huPFFs-injected mice and by 47–51% in mPFFs-injected mice (Fig 5a–b, Online resource 6b). However, there were no significant differences between huPFFs and mPFFs in the ipsi- and contralateral AON (Online resource 6b). Differences between ipsilateral and contralateral AON were not significant either (Online resource 6a) indicating that despite unilateral injection, both sides were affected similarly and severely at 6 months post-injection. At 18 months post-injection, huPFFs and mPFFs groups showed no further decrease in cell loss compared to 6 months (Fig 5b, Online resource 6c), suggesting that severe PFFs-induced cell loss occurred within 6 months after the injection only. Surprisingly, we observed a 50–77% decrease in total cell number

between 6 and 18 months in the control groups (mMs-injected and non-injected aged match controls) indicating cell loss in the AON due to aging (Fig 5b, Online resource 6c).

Since cresyl violet labels both neuronal and non-neuronal populations, we also quantified cells by their appearance: lightly stained cells (neuronal population, Fig 5c) and dark stained cells (which include glial cells, and small interneurons; Fig 4d) (for details, see Material and Methods). Both populations are affected by age and by the injectate; indicating pathology induced by PFFs injections does not affect a specific cell population.

We previously reported no loss of mitral cells 6 months post-injection [59]. Those findings indicated that the injection process itself and the α -syn pathology that was evident in the OB 1–6 months post-injection were not deleterious to mitral cells, which are the OB projection neurons that connect to secondary olfactory regions and are easily identified by their characteristic morphology (Fig 6a–b). To confirm that mitral cell loss in the OB is not simply delayed compared to cell loss we measured in the AON, we quantified the density of cresyl violet-positive mitral cells in the OB at 18 months post-injection (Fig 6). Again, we observed no significant differences in mitral cell density between the groups at 18 months, and the densities were in the same range (Fig 6c) as those we previously reported for mice at 6 months post-injection [59]. This confirms that it is unlikely that mitral cell loss in the OB contributes to the behavioral deficits we have previously described in this model [59].

Discussion

We performed carefully controlled microinjections of huPFFs and mPFFs assembled from recombinant α -syn monomers [58] to trigger α -synucleinopathy in the OB of wild-type mice. Building on our previous report where we observed progressive worsening of α -synucleinopathy and olfactory deficits over one year after injection, we now observe mild propagation following longer delays. At 18 and 23 months after injection, in general, α -syn inclusions appeared in additional brain regions compared to the results at 12 months, but that within several brain regions previously affected, the density of the α -syn pathology decreased. We also observed marked cell loss in the AON at 6 and 18 months.

Pser129 inclusions at extended time points post-injection are mature inclusions

As expected, the pser129 inclusions present 18 and 23 months post injection were located in neurons and detectable by an antibody against α -syn and Thioflavin S staining, confirming that they were mature. These inclusions had characteristics similar to those of inclusions 1 month post PFFs injection [59]. As mentioned earlier, in the AON we observed additional inclusions that were not co-localized with the NeuN marker. Since neuronal populations localized in the AON are all labelled by the marker NeuN [42, 66, 75], our observations suggest that non-neuronal cells can also contain α -syn inclusions at 18 months. This is consistent with the observation of inclusions in oligodendrocytes and astrocytes in synucleinopathies and with previous reports from cell culture and animal models (reviewed in [10]). An alternative explanation could be that these inclusions are localised in microglia that can phagocytose aggregates in PD [65]. Another possible explanation is that the cells bearing the inclusions are dying neurons that stopped expressing the NeuN marker [21, 32].

Increased number of brain regions are affected by α -synucleinopathy after 18 months but not after 23 months

We then assessed α -syn pathology 18 and 23 months post-injection of PFFs. Compared to mPFFs-induced pathology spreading observed between 1 and 12 months post-injection (Fig 3, [59]), five additional brain regions display pathology at 18 months (i.e. ipsilateral DP, RML, ipsilateral and contralateral IL, contralateral OB and Th). Among these regions, some have direct connections with the ipsilateral OB or with other olfactory regions (DP, IL; [72]), and thus could be expected to develop pathology at an earlier time point. Notably, the contralateral OB developed pathology only at 18 months, although it had received direct projections from the contralateral AON, which had already developed pathology at one month post-injection, and it receives feedback projections from other contralateral olfactory regions that developed pathology 3 months post-injection [59]. By contrast, some regions that contained α -syn pathology at 12 months post-injection of mPFFs [59] did not exhibit pser129 inclusions at 18 months post-injection (e.g. ipsilateral Th; association area of the temporal cortex, TeA; Substantia nigra, SN; Locus coeruleus, LC; contralateral entorhinal, Ent, and ectorhinal, Ect, cortex).

Compared to mPFFs, we previously showed that huPFFs induced less widespread pathology after 12 months [59]. Here, we demonstrate that huPFFs-induced pathology progresses further at 18 months, appearing in many additional brain regions including the ipsilateral Ins, S1, S2, M1, M2, LC, SN, RN, and contralateral OT, dentate gyrus, Cornu Ammonis 2, molecular layer of the hippocampus, and LH, thus reaching a spatial extent comparable to what was observed 12 months after mPFFs injections [59]. However, at 23 months post-injection, pathology does not progress further anatomically after huPFFs injection, and several brain regions that contained pathology at 18 months no longer display pathology (e.g. sensory and motor cortices, SN, basomedio- and lateral nuclei of the amygdala, CPu), suggesting a regression of the spatial extent of pathology. Brain regions that showed only transient pathology at 12 months post-mPFFs injection and 18 months post-huPFFs injection contained very mild pathology. These observations suggest that, in this model, particular brain regions that exhibited a small amount of pathology soon after injection may be able to clear pathological inclusions and stop the development of additional inclusions. The precise mechanism for the clearance of inclusions in our model is unknown, but we know from other studies that the cells themselves are able to degrade (parts of) inclusions via autophagy-lysosomal and ubiquitin-proteasome degradation systems [76]. This could contribute to a decrease of inclusions in specific brain regions in our model. In addition, cells containing inclusions might degenerate, as described previously in another PFF-model [47]. Finally, microglial cells are able to clear dead cells and debris (for review, see [10, 77] and astrocytes were shown to degrade α -syn aggregates in cell culture systems [35] suggesting that both of these cells types might contribute to clearing inclusions in our model. Finally, the effect of PFFs injection on the expression of endogenous α -syn in WT mice has not yet been investigated. It is conceivable that PFFs-related pathology could suppress expression of endogenous α -syn in our model and reduce the availability of endogenous α -syn to form aggregates. Supporting this idea, the work by Lim and colleagues [34] demonstrated that the suppression of α -syn expression for 3 months in an α -syn inducible

transgenic mouse model leads to a decrease in α -syn pathology in the brain and reverses synaptic defects and memory deficits.

In our study, while PBS-injected or non-injected groups did not exhibit pser129 inclusions, some mice that received injections of mMs (18-months post-injection: 2 mice out of 4; 23-months post-injection: 1 mouse out of 4) showed rare pser129-positive inclusions in some olfactory regions (see Fig 1a). Previously, we studied mMs-injected mice from 1 to 12 months, and did not observe pathology within this time range [59]. Others have reported the development of pser129-positive inclusions in cortical areas and the SN at 6 months after injection of non-fibrillized α -syn into the striatum of rats [49] or that soluble α -syn injected into transgenic mice overexpressing α -syn led to α -syn aggregation at 4 months post-injection [62]. It is possible that aggregation of mMs may have occurred in the test tube just prior to the injection or after injection, given the high concentration of monomers present in solution. Differences in the host (rat or transgenic mice in the other studies, versus wild-type mice in our studies) might explain why in our experiment mMs induced occasional pathology so late after the injection (18 months) compared to the previous literature (4 months).

Overall, our observations in this study and in our previous work [59] strongly support a propagation of pathology between brain regions connected via neuronal pathways. However, we cannot rule out that other mechanisms might be involved. An alternative explanation is the idea that all of the brain areas that eventually develop aggregates are exposed to PFFs immediately following PFFs injection, but develop pathology in a sequential manner. This hypothesis does not explain why pathology appears sequentially in interconnected brain regions originating from the injection site, and do not appear at early time points in regions that are not connected to the OB. It is also possible that PFFs injections trigger cellular dysfunction mechanisms that are transmitted to neighbouring neurons, instead of a transmission of α -syn seeds themselves; further work is needed to rule out this possibility. Finally, it is possible that cells that are able to migrate, such as microglia, might carry pathogenic seeds to distant brain regions, which has been suggested to occur in models of tauopathy [1]. However, in our model, we were unable to detect pser129 α -syn in microglia [59]; and if migrating cells were the vector of pathology spreading, we would expect appearance of pathology inversely proportional to distance from the injection site, and in regions that are not always directly and indirectly connected to the OB.

Decrease in the density of α -syn pathology 18 months post-injection

Although pathology progressed to additional brain regions at 18 months post-injection, the overall density of pathology in the brain decreased. While mPFFs triggered dense to severe pathology in several brain regions at 12 months post-injection [59], only mild to moderate scores were observed at 18 months post-injection. Densitometry of pser129 pathology in olfactory regions confirmed a marked decrease in pathology density between 12 and 18 months post-injection in the OB, the PC and the Ent after injection of mPFFs. In mice injected with huPFFs, moderate pathology was observed up to 18 months post-injection (confirmed with pser129 quantifications), while at 23 months, only mild pathology was present. Our observation of a decrease in the density of the pathology at later time points is

consistent with previous work. In a model of PFFs-induced α -syn pathology by injection of PFFs into the striatum of rats, Paumier and colleagues described a decrease in pathology in the substantia nigra 180 days post injection compared to earlier time points [49].

In our model, the pathology observed between 1 and 12-months post-injection was relatively severe in olfactory structures, and particularly after mPFFs injections [59]. We believe that neurons which contained pathology [47] before 12 months died, and were then degraded which resulted in an apparent decrease in the quantity of pser129-positive aggregates at 18 months. Our data show that injection of PFFs in WT mice induces α -syn pathology that progresses further in terms of spatial spreading until 18 months post-injection, but that the density of α -syn aggregate pathology does not increase after more than a year and declines later (the last time point studied was 18 months for mPFFs, and 23 months for huPFFs). Thus, to reproduce more closely the long-term rise in the severity of pathology in mouse models, as observed at late stages of PD, additional treatments beyond single PFFs injections appear to be necessary. Other unknown factors may contribute to the induction of α -syn pathology following injections of α -syn PFFs and they might be necessary to model the aging state of the human brain when sporadic PD pathology develops. For example, aged mice or those that display defects in protein clearance or in mitochondrial activity, or senescence accelerated-mice could be used.

Severe cell loss in the AON, but not the OB, following PFFs injection

In the same model of progressive α -synucleinopathy that we describe in this paper, we have previously reported olfactory deficits which progressively develop over 1–12 months [59]. We also previously described that there was no loss of mitral cells in the OB 6 months after injection of PFFs [59]. To determine if mitral cells undergo delayed degeneration in the OB, we assessed whether injection of PFFs led to a loss of neurons after 18 months. We found no decrease in mitral cells density at 18 months post-injection, indicating that mitral cells are resistant to PFFs-induced insults despite the injection being made directly into the OB and these cells being the first cells affected by the pathogenic α -syn fibrils [59].

We next examined if there was cell loss in the AON that could potentially contribute to the olfactory deficits we previously reported [59]. A shortcoming with our approach when we quantified the numbers of cresyl violet-stained cells in the AON is that we could not distinguish small neurons from glia. While cresyl violet-positive large pyramidal neurons of the AON can easily be identified by morphology (cresyl violet-positive, large faint cells that we quantified), our approach does not allow us to differentiate glial cells from small interneurons or small pyramidal neurons that exhibit similar morphologies (cresyl violet-positive, small dark cells). However, statistical analyses demonstrate that the large faintly cresyl violet-positive cells (large pyramidal neurons) and the small dark cresyl violet-positive cells (mix of glial cells and small neurons) are affected by the injectate and by aging in a similar way.

Our stereological counting in the AON revealed severe loss of cresyl violet-positive large pyramidal neurons 6 months after injection of huPFFs and mPFFs. In the AON, 40 to 60% of the cresyl violet-stained cells disappeared, a magnitude in the same range as the loss of AON neurons observed in PD patients [50]. At 18 months post-injection, we observed a

decrease in AON cell number in control animals (including in aged-matched non-injected animals) compared to young mice, indicating an effect of normal aging. No further cell loss was observed in PFFs injected animals at 18 months. PFFs injection precipitate cell loss in the AON by 12 months. Data on loss of cells in the AON during aging is sparse as one study demonstrates a decrease of neuropeptide Y neurons with age [25]. The lack of further cell loss in the AON at 18 months post injection suggests that a specific cell population in the AON was affected at 6 months, and that this same population eventually succumbs as a result of normal aging in our control mice. A recent study suggests that α -syn PFFs can trigger mitochondrial dysfunction in a specific subpopulation of cultured cells [68], and it is conceivable that a similar mechanism contributes to neuronal death in our *in vivo* paradigm. Further studies are required to precisely define which neurons die, and to what extent different neuronal subtypes (e.g. interneurons versus projection neurons) are selectively vulnerable in the AON and other olfactory regions in this mouse model. Finally, studies that define the neuroinflammatory response are needed to determine its contribution to propagation of α -syn aggregates and cell loss.

Conclusions

We demonstrate that injection of α -syn PFFs into OB triggered progressively increasing α -syn pathology, followed by severe cell loss at 6 months post-injection and that pathology decreased in severity with slight anatomical progression at 18 months in our model. The former indicates that at early time points our model of prodromal PD can be used to identify compounds that halt the propagation of α -syn pathology and prevent cell loss. Altogether, our previous and present work demonstrate that our paradigm is suited for modelling olfactory-related pathology at prodromal stages between 1 and 12 months. Further studies are needed to explore the long-term effect of injections of α -syn PFFs in mice with alterations that mimic those associated with increased PD risk (e.g. mitochondrial deficits, neuroinflammation).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in the text of the article

AON	anterior olfactory nucleus
α-syn	alpha-synuclein
BLA, BLP, BLV	basolateral amygdala
CeL, CeL, CeC, CeA	central amygdaloid nucleus of the amygdala
DP	dorsal peduncular cortex
Ect	ectorhinal cortex
Ent	entorhinal cortex
Hipp	hippocampus
huPFFs	pre-formed fibrils of recombinant mouse α -synuclein
IL	infra-limbic cortex
Ins	insular cortex
LC	locus coeruleus
LH	Lateral hypothalamic area
LMMs	linear mixed-effects models
M1,M2	primary and secondary motor cortex
mMs	mouse monomeric α -synuclein
mPFFs	pre-formed fibrils of recombinant mouse α -synuclein
OB	olfactory bulb
PC	piriform cortex
PD	Parkinson's disease
PLH	peduncular part of the lateral hypothalamic area
PFFs	pre-formed fibrillar assemblies of recombinant α -synuclein
Pser129	α -syn phosphorylated on serine 129
PVA	paraventricular thalamic nucleus, anterior part
RML	retro mammillary nucleus, lateral part
RN	raphe nucleus
S1,S2	primary and secondary somatosensory cortex
SN	substantia nigra

TeA	association area of the temporal cortex
V2	secondary visual cortex

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Figure 1. PFFs-induced pathology at 18 and 23 months post-injection is mild

(a) α -Syn pathology detected by an antibody against pser129 is present in brain regions ipsilateral (legend in blue) and contralateral (legend in red) to the injection site. Diagrams illustrating the anatomical location of α -syn pathology (assessed by antibody against pser129) in the brain on coronal sections at 18 months post-injection of mPFFs (b), of HuPFFs (c) and at 23 months post-injection of HuPFFs (d). The green star indicates the injection site. Severity of pathology is represented by different colours, from no pathology to severe pathology and is based on a scoring method. The sections were immunostained in 3 independent histochemical experiments. Histochemical analysis was performed on all animals at 18-months post-injection of mPFFs, and at 23-months post-injection of HuPFFs, mMIs and PBS as well as in 21 and 26 months-old non-injected animals (age-matched; PBS-injected and non-injected mice not shown) (mPFFs 18 months: n=3; HuPFFs 18 months:

n=5, HuPFFs 23 months: n=4, mMs 18 months and 23 months: n=4 for each time point; PBS 18 months: n=3); non-injected mice 18 months post-injection time (age: 21 months-old): n=3; 23 months post-injection time (age: 26 months-old): n=3). The data presented here are from representative animals. Scale bar: 20 μm . All the images with a purple scale bar were acquired at 60x; all the other images were acquired at 40x and match the black scale bars. A list of brain structure abbreviations is available in Online Resource 4.

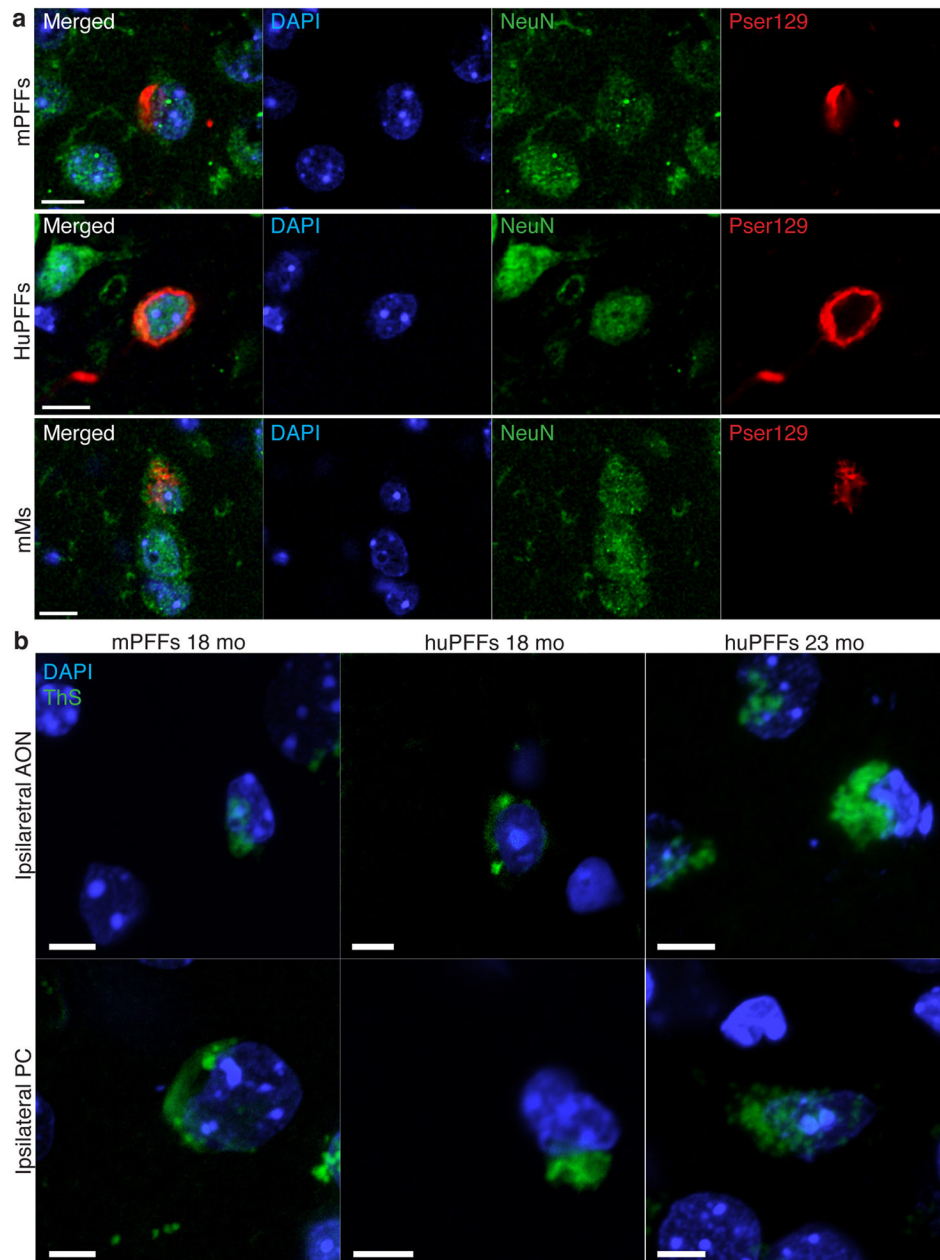


Figure 2. Pser129-positive inclusions induced by injected PFFs or mMs are localized within neurons, and thioflavin-S positive
 a) Pser129-positive inclusions (red), NeuN-positive cells (green), and nuclei stained by DAPI (blue) in the ipsilateral AON 18 months after injection of mPFFs, HuPFFs or mMs. The sections were immunostained in 2 independent histochemical experiments. Histochemical analysis was performed on 3 animals per group 18-months post-injection of mPFFs, HuPFFs and mMs. b) Images from confocal stacks showing examples of Thioflavin-S positive inclusions detected in the ipsilateral AON and PC of PFFs-injected mice. 3 animals per group (mPFFs 18 months, huPFFs 18, and 23 months) were stained and analysed in a single experiment. Scale bars: a) 10 μ m; b) 5 μ m

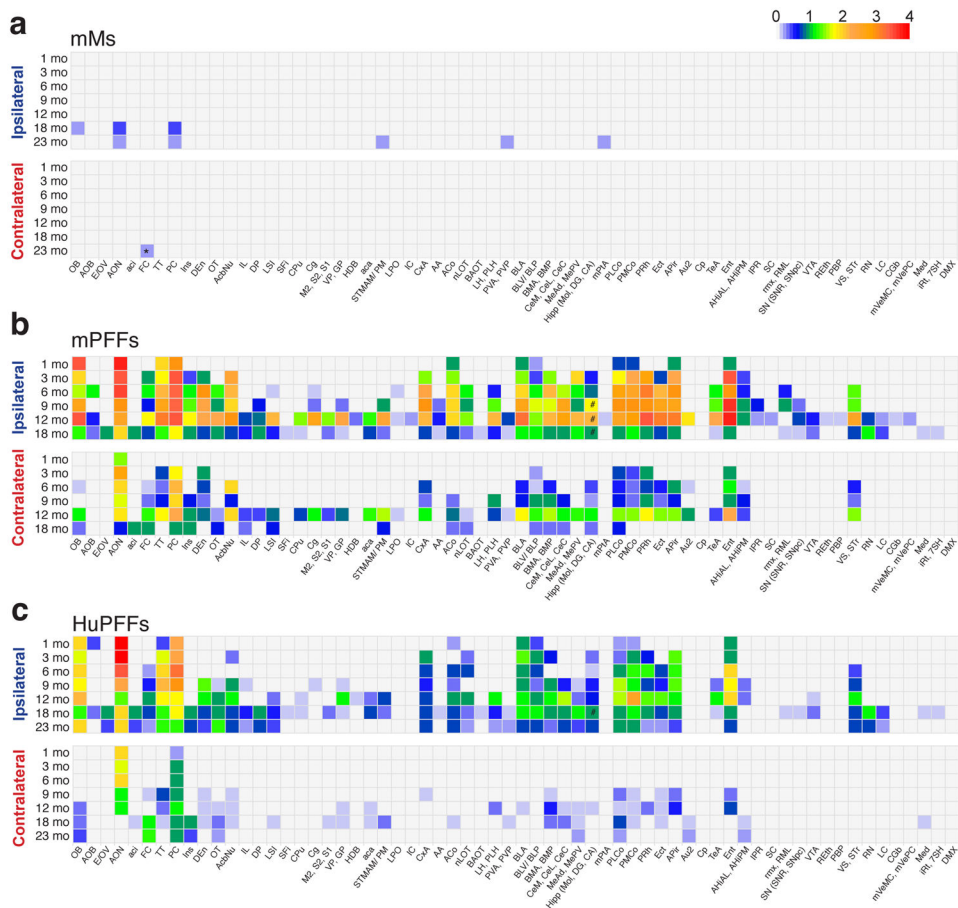


Figure 3. PFFs-induced pathology propagates 18 months after injection

Heatmaps showing the severity of pathology (on a scale from 0 to 4; data shown as the mean score of each group) in various brain regions ipsilateral (legend in blue) and contralateral (legend in red) to the site of injection, from 1 to 23-months post-injection of mMs (a), from 1 to 18 months post-mPFFs injection (b) and from 1 to 23-months post-injection of HuPFFs (c).

The scoring was performed on all animals at 9, 18-months post-injection of mPFFs, and at 23-months post-injection of HuPFFs, mMs and PBS as well as in 12, 21 and 26 months-old non-injected animals (age matched- controls; PBS-injected and non-injected mice not shown) (mPFFs 9 months: n= 5, HuPFFs 9 months: n= 5, mMs 9 months: n=5, PBS 9 months: n=4; mPFFs 18 months: n=3; HuPFFs 18 months: n=5, HuPFFs 23 months: n=4, mMs 18 months and 23 months: n=4 for each group; PBS 18 months: n=3, non-injected mice 21 months-old (aged-matched to 18 months post-injection groups): n=3; non-injected mice 26 months-old (aged-matched to 23 months post-injection groups): n=3). For 1, 3, 6, and 12 months delays, the average scores were calculated from analysis on animals presented in our previous article (delay 1, 3, 6, 12 months, [59] (n=4–5 animals per group). All the animals (1 to 23-months delays) are part of the same experiment (divided in 4 different experimental sessions). *Mild pathology is observed on contralateral frontal cortex in only 1 mMs-injected mouse out of 4, at 23 months post-injection. # At extended time points, the ipsilateral Hipp exhibits pathology in the radial, molecular, pyramidal, oriens

layers, the cornu ammonis, dentate gyrus, and the pyramidal and molecular layer of the dentate gyrus). A list of brain structure abbreviations is available in Online Resource 4.

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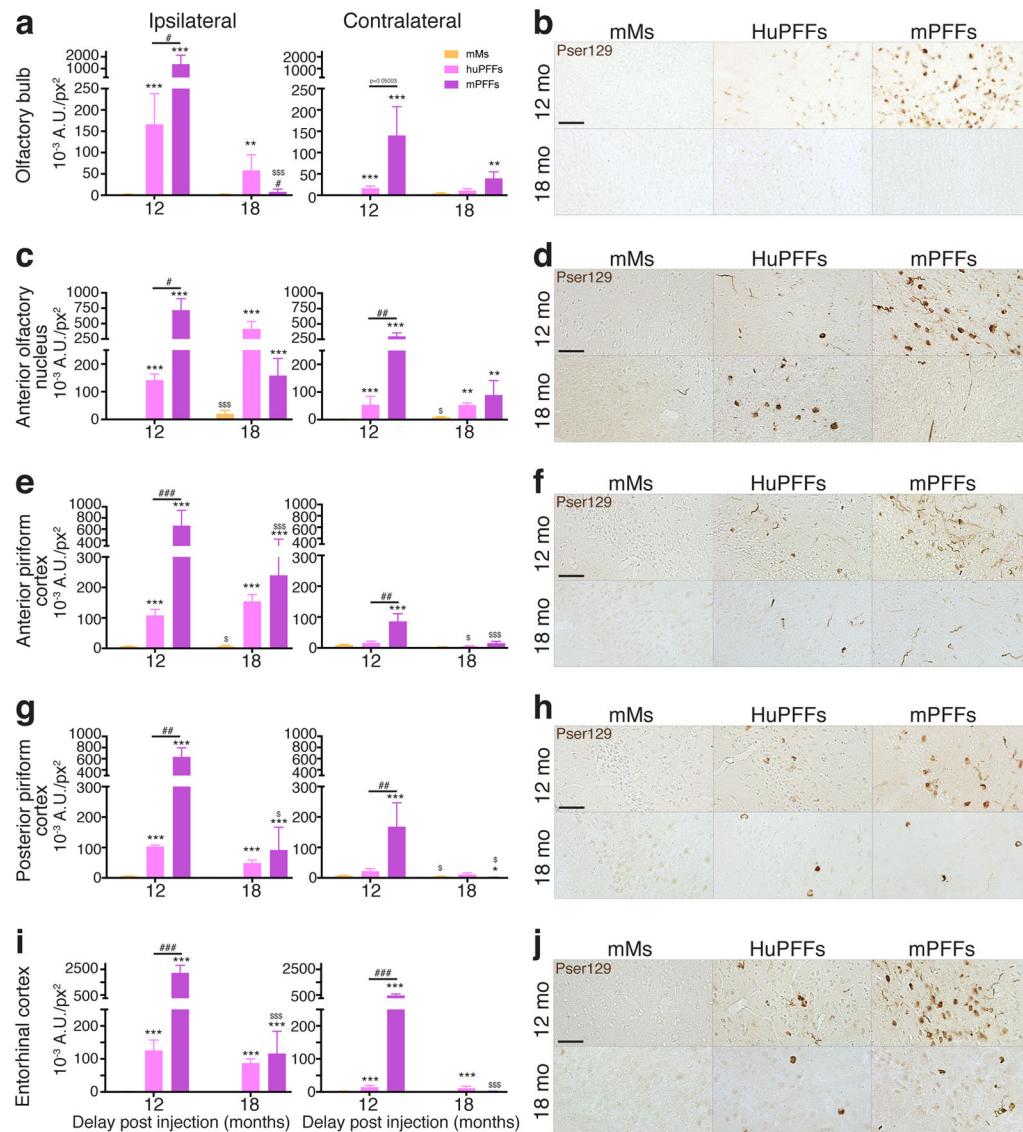


Figure 4. Pathology load decreases 18 months post-injection in specific brain regions

Densitometry of α -syn pathology (pser129 staining) measured in ipsi- and contralateral olfactory bulbs (a), anterior olfactory nuclei (c), anterior piriform (e), posterior piriform (g) and entorhinal cortices (i) at 12 and 18 months post-injection of mMs, HuPFFs and mPFFs. The sections were immunostained in 7 independent histochemical experiments. Representative images of the average densitometry of pathology in the olfactory bulb (b), anterior olfactory nucleus (d), the anterior piriform (f), posterior piriform (h) and entorhinal (j) cortices. Densitometry of pser129 pathology in the OB, AON, anterior and posterior PC and Ent was measured in all animals at 12 and 18 months post-injection of mMs, HuPFFs or mPFFs (mPFFs 12 months: n=4; mPFFs 18 months: n=3; HuPFFs 12 months: n=5; HuPFFs 18 months: n=5; mMs 12 months: n=5; mMs 18 months: n=4). For the PC, the anterior PC and the posterior PC were examined separately, and no significant difference between them was observed. Statistical analyses were performed by linear mixed-effect models (regression

and contrasts) to include three factors: delay post-injection, ipsi/contralateral and injectate. Statistical data are available in Online Resource 5.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to mMs injected mice at a given time point and side of the brain. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ for comparisons between mPFFs and huPFFs injected mice at a given time point and side of the brain. \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ for comparison between 12 and 18 months delays for a given mouse group and side of the brain. Scale bar: 50 μm

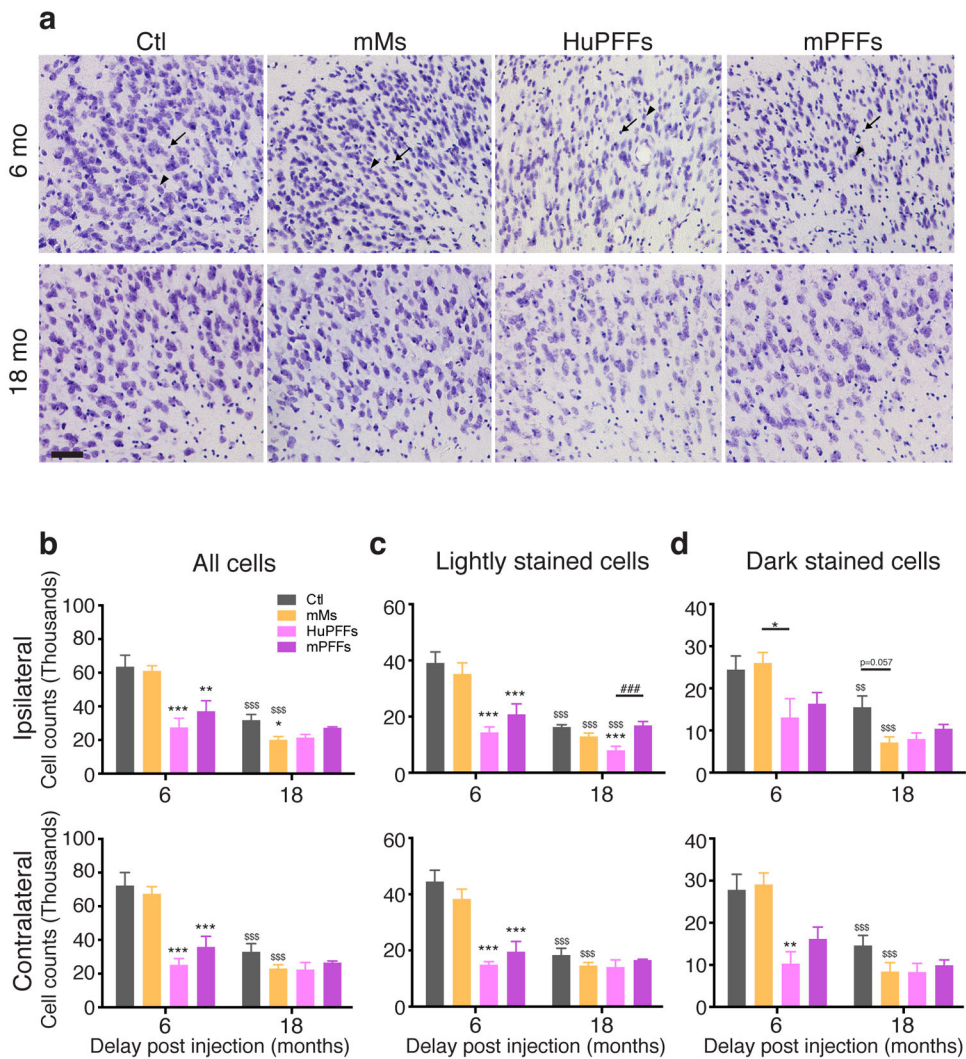


Figure 5. Cell loss in the AON measured by stereology

(a) Representative images of cresyl violet staining in the AON of mMs, HuPFFs and mPFFs injected mice, 6 and 18 months post-injection and in aged matched non-injected mice (Ctl; 9 and 21 months-old). (b–d) Cresyl violet-positive cell counts assessed by stereology in the ipsilateral and contralateral AON of these mice. (b) Total number of cresyl violet-positive cells. (c) Subpopulation of large cells (identified by arrowheads in (a)). (d) Subpopulation of small dark cells (identified by arrows in (a)). Data were analysed by negative binomial mixed-effects model to include three factors: delay post-injection, ipsi/contralateral side and injectate. (a–d) The sections were stained in 5 independent histological experiments. Stereological analysis was performed on all animals at 6 months and 18 months post-injection of mPFFs, HuPFFs and mMs (6 months post-injection: mMs: n=5, mPFFs: n=5, HuPFFs: n=4, 18 months post-injection: mMs: n=4, mPFFs: n=3, huPFFs: n=5), and in aged-matched non-injected mice (n=3 per age). Statistical data are available in Online Resource 6. * p<0.05, ** p<0.01, ***p<0.001 in comparison to mMs-injected mice and to non-injected mice at a given time point and a given side of the brain. # p<0.05, ## p<0.01, ### p< 0.001 for comparisons between mPFFs- and huPFFs-injected mice at a given time

point and side of the brain. \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ for comparison between 12 and 18 months delays for a given mouse group and a given side of the brain. Scale bar: 50 μm .

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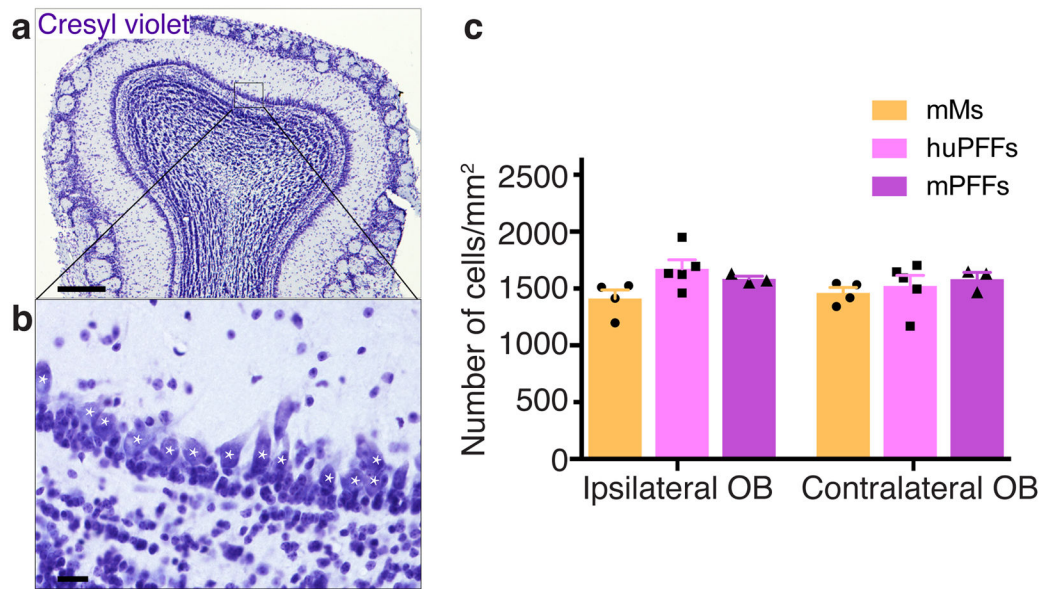


Figure 6. No loss of mitral cells in the OB at 18 months post-injection of PFFs

(a–b) Cresyl violet staining in the OB from one representative animal at low magnification (a), and high magnification (b) showing mitral cells (cell bodies identified by white stars).

(c) Density of mitral cells in the mitral layer of the OB 18 months post-injection of HuPFFs, mPFFs and mMs. We found no significant evidence of heteroscedasticity using a Brown-Forsythe test. We then performed two-way ANOVA with repeated measures where no significant effect was observed. (Group effect: $F(2,9)=1.840$ $p=0.2138$, Ipsi/contralateral effect: $F(1,9)=0.6184$ $p=0.4519$, Interaction: $F(2,9)=2.082$ $p=0.1807$).

The sections were stained in 2 independent histological experiments. Stereological analysis was performed on all animals 18-months post-injection of mPFFs, HuPFFs and mMs.

(mMs: $n=4$, mPFFs: $n=3$, HuPFFs: $n=5$).

Scale bars: (a) 500 μm ; (b) 20 μm .