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# 1 Identification of distinct pathological signatures induced by patient-derived $\alpha$ - 2 synuclein structures in non-human primates 3 4

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73 **ABSTRACT**

74 Dopaminergic neuronal cell death, associated with intracellular  $\alpha$ -synuclein ( $\alpha$ -syn)-rich protein  
75 aggregates (termed ‘Lewy bodies’), is a well-established characteristic of Parkinson’s disease.  
76 Much evidence, accumulated from multiple experimental models has suggested that  $\alpha$ -syn plays a  
77 role in PD pathogenesis, not only as a trigger of pathology but also as a mediator of disease  
78 progression through pathological spreading. Here we have used a machine learning-based approach  
79 to identify unique signatures of neurodegeneration in monkeys induced by distinct  $\alpha$ -syn pathogenic  
80 structures derived from PD patients. Unexpectedly, our results show that, in non-human primates,  
81 a small amount of singular  $\alpha$ -syn aggregates is as toxic as larger amyloid fibrils present in the LBs,  
82 thus reinforcing the need for preclinical research in this species. Furthermore, our results provide  
83 evidence supporting the true multifactorial nature of PD as multiple causes can induce similar  
84 outcome regarding dopaminergic neurodegeneration.

85

86

87 **INTRODUCTION**

88

89 The seminal work of Braak and colleagues suggesting that Lewy body (LB) pathology follows a  
90 predictable pattern of progression within the brain in Parkinson's disease (PD) (1) as well as the  
91 'host-to-graft' observation (2-4) led to the development of experimental models based on injection  
92 with  $\alpha$ -synuclein ( $\alpha$ -syn – the primary protein component of LB) assemblies (5-7). These  
93 experimental models suggest that  $\alpha$ -syn, in pathological conformations such as the one found in  
94 LBs, initiates a cascade of events leading to dopaminergic neuron degeneration as well as cell-to-  
95 cell propagation of  $\alpha$ -syn pathology through a self-templating mechanism.

96 Several studies have suggested that pre-fibrillar oligomers may represent one of the major  
97 neurotoxic entities in PD (8, 9). This notion has been derived primarily from studies using large  
98 doses of recombinant  $\alpha$ -syn applied to cell cultures or injected into adult mice, over-expressing  
99 either mutant or wild-type  $\alpha$ -syn (10). In agreement with these findings, we have shown that  
00 intracerebral injection of low doses of  $\alpha$ -syn-containing LB extracts, purified from the substantia  
01 nigra, pars compacta (SNpc) of postmortem PD brains, promotes  $\alpha$ -syn pathology and  
02 dopaminergic neurodegeneration in wild-type mice and non-human primates (11). Importantly, this  
03 neuropathological effect was directly linked to the presence of  $\alpha$ -syn in LB extracts, since immuno-  
04 depletion of  $\alpha$ -syn from the LB fractions prevented the development of pathology following  
05 injection into wild-type mice.

06 In this study, our aim was to thoroughly investigate this experimental model of synucleinopathy in  
07 non-human primates. The initial study design was to administrate fractions derived from the same  
08 PD patients containing either soluble and small  $\alpha$ -syn aggregates (hereafter named noLB) or LB-  
09 type aggregates (hereafter named LB). However, because of the unexpected finding that non-  
10 human primates, unlike mice, are susceptible to soluble or finely granular  $\alpha$ -syn, we sought to  
11 elucidate the response characteristics induced by either LB or noLB fractions. To achieve a  
12 thorough analysis of these  $\alpha$ -syn-related characteristics, we took advantage of the strength of  
13 machine-learning algorithms for discovering fine patterns among complex sets of data and  
14 developed a new method compatible with the constraints of experimental biology. We here report  
15 the identification of primate-specific responses to selected  $\alpha$ -syn assemblies associated with  
16 different pathogenic mechanisms. Overall, our results support the concept of the multifactorial  
17 nature of synucleinopathies.

18

19

## 20 RESULTS

### 21 *Purification and characterization of $\alpha$ -synuclein extracts from PD patients*

22 NoLB and LB fractions were obtained from the SNpc of five sporadic PD brains exhibiting  
23 conspicuous LB pathology. The samples were processed through differential ultracentrifugation in  
24 a sucrose gradient, and analyzed for the presence of  $\alpha$ -syn aggregates by filter retardation assay  
25 (Fig. 1A) (11). Further characterization of noLB and LB fractions was performed by co-localization  
26 of  $\alpha$ -syn and the amyloid dye Thioflavin S (Fig. 1B) as well as ultrastructural examination by  
27 electron microscopy (Fig. 1C). These assays confirmed the presence of misfolded  $\alpha$ -syn in both  
28 fractions. We also performed biochemical characterization of the stability of assemblies after  
29 proteinase K digestion (Fig. 1D) and detergent treatments (Fig. 1E) followed by  $\alpha$ -syn dot-blot  
30 assays. While total  $\alpha$ -syn content was comparable between selected fractions (as measured by  $\alpha$ -  
31 syn ELISA), LB fractions showed higher resistance to proteinase K treatment (noLB  
32  $t_{1/2}$ =15.23minutes vs LB  $t_{1/2}$ >60minutes) (Fig. 1D) as well as greater resistance to multiple  
33 detergents, including 8M Urea (Fig. 1E). We then measured the content of  $\alpha$ -syn aggregates using  
34 human  $\alpha$ -syn aggregation TR-FRET-based immunoassay, which revealed a significantly higher  
35 amount of aggregated  $\alpha$ -syn in LB fractions (Fig. 1F). To obtain insight into the content of  
36 monomeric and aggregated  $\alpha$ -syn within noLB and LB fractions of PD patients, sarkosyl treatment  
37 was applied to both fractions to induce physical separation, and then velocity sedimentation and  
38 density floatation gradients were performed to quantify these two respective populations and  
39 determine their relative abundance in each fraction (Fig. S1 A-H). Strikingly, while LB fractions  
40 contained ~90% of aggregated  $\alpha$ -syn, noLB fractions were composed of ~10% of this pathological  
41 form of the protein (Fig. S1 I). Also, in order to confirm the quality of the LB extraction, we  
42 performed a filter retardation assay which showed that LB fractions, but not noLB fractions, were  
43 highly enriched in known components of LBs, such as phosphorylated S129  $\alpha$ -syn, ubiquitin, p62,  
44 hyperphosphorylated tau and A $\beta$  (Fig. S2 A).

45 Micro-Infrared Spectroscopy of LB and noLB fractions was performed to show conformational  
46 changes in amyloid structures at the molecular level (Fig. S2 B-E) and this confirmed the presence  
47 of  $\beta$ -sheet structures in both assemblies (Fig. S2 B-C). Although their velocity of sedimentation  
48 and density floatation characteristics were similar, the aggregates present in the LB and noLB  
49 fractions were different in nature based upon the evidence of Micro-Infrared Spectroscopy.  
50 Principal component analysis (PCA) showed that, in the LB fractions, large aggregates  
51 corresponding to the major pieces of LB were present (Fig. S2D, cluster on the right). PCA further  
52 showed that, in the range of 1,590-1,700  $\text{cm}^{-1}$ , the LB group contained a fraction of amyloid

53 aggregates with different amyloid structures from those in the noLB group as they clearly  
54 segregated by PCA in two clusters (Fig. S2 D-E). Altogether, these results suggest that while LB  
55 fractions primarily contained large aggregated  $\alpha$ -syn fibrils, noLB fractions contained soluble  $\alpha$ -  
56 syn and a smaller enrichment of  $\alpha$ -syn aggregates featuring a specific amyloid structure not found  
57 in the LB fractions.

58 Data from several studies suggest that both recombinant  $\alpha$ -syn preformed fibrils (12-14) and  
59 patient-derived  $\alpha$ -syn (11) can promote pathogenic templating of endogenous  $\alpha$ -syn ultimately  
60 leading to dopaminergic neurodegeneration in SNpc. Following quantification by ELISA, both  
61 mixes of fraction were diluted to ~24 pg  $\alpha$ -syn per microliter. Then, those fractions were tested for  
62 their pathogenic effects on TH-positive dopaminergic neurons in primary mesencephalic cultures  
63 (Fig. S3 A) as well as *in vivo* in wild-type mice. Four months after supranigral injection, LB-injected  
64 mice displayed, as expected, significant dopaminergic degeneration, while noLB injections in mice  
65 had no impact on dopaminergic neurons (Fig. 1G-H) as we have previously reported for other  
66 SNpc-derived LB fractions (11), thus validating the toxicity of the preparation prior to injection  
67 into non-human primates.

68

69 *Intrastriatal injection of LB and noLB fractions from Parkinson's disease patients induces*  
70 *nigrostriatal neurodegeneration in baboon monkeys*

71 To determine the mechanisms of  $\alpha$ -syn aggregates toxicity in a species closer to humans, adult  
72 baboon monkeys (n=4-7 per experimental group) received bilateral stereotaxic injections (100 $\mu$ l)  
73 of either LB or noLB fractions into the putamen before euthanasia 24 months post-injection. This  
74 time-frame was chosen based on our previous studies indicating that after 14 months post-injection,  
75 ongoing pathogenic effects can already be measured, and was extended to potentially reach disease-  
76 relevant lesions. Two years after administration, LB-injected monkeys displayed significant striatal  
77 dopaminergic terminal loss both in the putamen and in the caudate nucleus, accompanied by a  
78 significant decrease in tyrosine hydroxylase (TH) immunoreactivity in the substantia nigra pars  
79 compacta (SNpc) (Fig. 2). Stereological counts showed that LB-injected animals exhibited TH-  
80 positive and Nissl-positive cell loss in the SNpc (16% and 23%, respectively). No overt  
81 parkinsonism was observed, however, since the extent of the lesion remained below the threshold  
82 for symptom appearance; i.e. 45% of cell loss (15), compared to an age-matched control group.

83 At odds with mice either generated for the purpose of this study (Fig. 1G-H), previously published  
84 (11), or produced in the context of other in-house studies (data not shown), noLB-injected monkeys  
85 showed degeneration of the nigrostriatal pathway including dopaminergic cell loss (i.e. 16% of TH-

86 positive neurons and 28% of Nissl-positive neurons quantified by stereology), similar to that  
87 observed in LB-injected monkeys (Fig. 2). Facing such an unexpected finding, we aimed to identify  
88 specific characteristics of the pathological mechanisms involved in  $\alpha$ -syn toxicity induced by each  
89 fraction independently, using a large-scale approach in combination with machine learning for  
90 pattern identification.

91

### 92 *Machine-learning algorithm predicts nigrostriatal degeneration*

93 We performed an exploratory approach and aimed to distinguish relevant variables allowing  
94 accurate prediction of neurodegeneration (i.e., to operate a feature selection). Overall, we  
95 investigated a large number of variables tapping on behavioral, histological, biochemical,  
96 transcriptional and biophysical approaches (Fig. 3A) applied to several brain areas (n=40 – Fig.  
97 3B), totalizing 180 variables measured for each individual (Fig. S4A for variable abbreviation  
98 nomenclature; Table S1 for exhaustive list of variables; Table S2 features all raw data). We first  
99 extracted from this dataset, every variable that actually quantified neurodegeneration (i.e.  
00 dopaminergic markers such as TH or dopamine transporter by immunohistochemistry), ending up  
01 with 163 variables per animal.

02 Then, to operate feature selection, we designed a distributed algorithm using multiple layer  
03 perceptron (MLP) (Bourdenx and Nioche, 2018), a classic machine-learning algorithm based on  
04 artificial neural network that is able to approximate virtually any functions (Hornik et al., 1989).  
05 This algorithm was given, as input, the data obtained for each animal for the 163 aforementioned  
06 variables and its output is a rank of these variables regarding their ability to predict three indicators  
07 of dopaminergic tract integrity; that were levels of tyrosine hydroxylase staining in (i) the SNpc,  
08 (ii) the putamen and (iii) the caudate nucleus.

09 The main difficulty was to overcome the large number of input variables (163) compared to the  
10 sample size (n=4-7 per group), which can induce a selection and reporting bias (Kuncheva and  
11 Rodriguez, 2018). In order to tackle this “ $p > n$ ” problem, instead of using a single network that  
12 could be prone to overfitting, we put in competition several networks.

13 Each MLP was composed of a single hidden layer of 3 neurons (Fig. 3C). It has as input a subset  
14 of 3 variables (out of the 163) and as output the 3 indicators of dopaminergic tract integrity. In total,  
15 we used 708,561 sets of 3 inputs variables. Every instance of MLP was trained with 80% of our  
16 sample (always a combination of control and injected animals) and tested on the remaining 20%.  
17 The performance of each set of 3 input variables was evaluated according to the difference between  
18 the predicted values of TH staining and the actual ones.



19 We focused on the top 1% of the best networks and counted the occurrence of each of the 163  
20 variables in the subset of 3 variables used by these best networks (Fig. 3C). We ranked each variable  
21 according to the number of occurrences (Fig. 3C) for LB- (Fig. 3D) and noLB-injected animals  
22 (Fig. 3E) independently.

23 In order to avoid possible overfitting, we used several methods in combination. First, we performed  
24 cross-validation by splitting the dataset into two parts: a training and a testing set of data. 80% of  
25 the data were randomly selected to train the networks (and independently for each network), while  
26 the 20% remaining were used to evaluate the networks. Then, in order to evaluate the robustness of  
27 the quality of prediction for a given set, we repeated this cross-validation step 50 times for every  
28 set of 3 input variables (each network was trained and tested using a different partition of the dataset  
29 - total number of network: 35,428,050). Lastly, we generated random data and used them as input  
30 for the MLP. As expected, performances were significantly lower compared to our actual dataset  
31 (Fig. S4B, C).

32 Overall, this unique approach allowed us to rank input variables according to their explanatory  
33 power and therefore to extract the strongest predictors of neurodegeneration for each experimental  
34 group. Interestingly, despite similar levels of nigrostriatal degeneration between LB- and noLB-  
35 injected animals (Fig. 2B), the algorithm allowed us to identify differential variable sorting patterns  
36 (Fig. 3D-E).

37

### 38 *MLP-derived signatures can identify unique characteristics between experiment group*

39 Next, we compared the LB and noLB characteristics using the rank-rank hypergeometric overlap  
40 (RRHO) test (Fig. 4A). Interestingly, low similarity was observed for the highly ranked variables  
41 suggesting specific differences in the biological response to the injection of LB or noLB (Fig. 4B).  
42 Focusing on the 20 first variables that showed low similarity between groups, we found that LB-  
43 exposed monkeys were characterized by both quantitative and qualitative changes in  $\alpha$ -syn levels  
44 (i.e. phosphorylation at Ser129 and aggregation) especially in cortical areas corroborated by distinct  
45 methodologies as well as by a dysfunctional equilibrium in neurochemistry of basal ganglia output  
46 structures classically associated with parkinsonism (16, 17) (Fig. 4C – Fig. S5). Conversely, noLB-  
47 exposed monkeys exhibited more diverse nigrostriatal-centric characteristics with variables related  
48 to  $\alpha$ -syn aggregation, proteostasis and Zn homeostasis (Fig. 4D - Fig. S6). Together, we identified  
49 specific properties for both groups with limited overlap (35% - 7/20 variables) for an identical level  
50 of degeneration.

51

### 52 *Retrospective literature search validates MLP derived signatures*

53 We next used a retrospective analysis to validate the relevance of the MLP-derived signature in  
54 PD. Although, some variables have never been investigated in the context of PD, others have been  
55 studied and reports exist in the literature. For instance, the amount of phosphorylated Ser129  $\alpha$ -  
56 synuclein in the entorhinal (*h.psyn.ctx.er.ant*) and parahippocampal (*h.psyn.ctx.phipp*) cortex - 1<sup>st</sup>  
57 and 2<sup>nd</sup> best predictors for the LB group – have been already associated with PD pathology. Studies  
58 of post-mortem brains from PD patients revealed the presence of LB in these regions which was  
59 correlated with disease progression (18) and predicted cognitive deficit in PD patients (19).  
60 Interestingly, the anterior entorhinal cortex has also been shown to be affected by severe  $\alpha$ -syn  
61 pathology, related to olfactory dysfunction in prodromal phases of PD pathology (20). In addition,  
62 increased levels of phosphorylated Ser129  $\alpha$ -syn in sensorimotor (*h.syn.ctx.sma.ant*) and  
63 cingulate cortices (*h.syn.ctx.cg.ant*), shared by both LB and noLB signatures, have already been  
64 reported by our group in an independent cohort of non-human primates (11).

65 Both LB and noLB signatures, and especially noLB, showed that variables related to  $\alpha$ -syn  
66 aggregation status were among the best predictors (LB: 1 in top10 best predictors; noLB 3 in top10  
67 best predictors). This was highly expected from the literature as  $\alpha$ -syn aggregation has been  
68 associated with PD pathology (21).

69 Variables related to the proteostasis network (levels of the lysosomal receptor LAMP2 –  
70 *wb.lamp2.sn* - 6<sup>th</sup> or amount of ubiquitinated proteins – *wb.ub.sn* – 9<sup>th</sup>) were more specifically  
71 associated with the noLB signature. This is of high interest as proteostasis defect is more and more  
72 considered as a key step in pathogenicity (22-24).

73 Levels of the microglia marker, Iba1, was ranked as the third best predictor of neurodegeneration  
74 in the LB signature. Microglial inflammatory response was shown to be implicated in  
75 neurodegeneration in many animal models, including  $\alpha$ -syn overexpressing and toxin-based animal  
76 model of PD (25).

77 Lastly, *postmortem* analysis of Zn<sup>2+</sup> concentration in the brains of PD patients has shown elevated  
78 levels in the striatum and SNpc (26). Conversely, a recent meta-analysis showed a decrease of  
79 circulating Zn<sup>2+</sup> levels in PD patients (27). In experimental models of PD, Zn<sup>2+</sup> accumulation has  
80 been associated with dopaminergic degeneration in rodent exposed to mitochondrial toxins (28,  
81 29).

### 82 83 *Experimental confirmation of MLPs' prediction*

84 We aimed to confirm the relevance of the top first MLP selected variables. Since the LB signature  
85 was associated with changes in  $\alpha$ -syn phosphorylation in cortical areas, we analyzed side-by-side  
86 the levels of  $\alpha$ -syn and phosphorylated Ser129  $\alpha$ -syn in 18 brain regions (Fig. 5A). Interestingly,

87 in agreement with the LB signature obtained from the MLP, LB-injected monkeys displayed a  
88 stronger accumulation of phosphorylated Ser129  $\alpha$ -syn compared to noLB-injected animals (Fig.  
89 5A-B). Also, the 2 most enriched variables of the LB signature (i.e. phosphorylated  $\alpha$ -syn levels in  
90 parahippocampal and entorhinal cortices (Fig. 4C)) showed significant negative correlations with  
91 degrees of degeneration (Fig. 5C-D), thus confirming their ability to predict neurodegeneration.  
92 Then, we decided to confirm the relevance of one of the strongest predictors, the levels of  $Zn^{2+}$  in  
93 the SNpc in independent experiments. First, we observed a significant increase of  $Zn^{2+}$  in noLB-  
94 injected mice compared to sham-injected or LB-injected mice (Fig. S7A). Second, we analyzed the  
95 levels of  $Zn^{2+}$  in LB-injected macaque monkeys from a previous study of our laboratory (11).  
96 Interestingly, despite the fact that these experiments were done in a different non-human primate  
97 sub specie, injection of LB in the putamen (similar to the present study) or above the SNpc (different  
98 from the present study) induced elevation of  $Zn^{2+}$  levels in the SNpc, as measured by SR-XRF (Fig.  
99 S7B). Of note, the dimension of the effect was similar across studies (Fig. S7E). Then, to  
00 understand whether that modulation  $Zn^{2+}$  levels was specific to our experimental paradigm, we  
01 measured  $Zn^{2+}$  levels in the context of adeno-associated virus-mediated overexpression of mutant  
02 human  $\alpha$ -syn in both rats and marmoset monkeys (30) using the same methodology (Fig. S7C, D).  
03 Here, overexpression of  $\alpha$ -syn did not triggered accumulation of  $Zn^{2+}$  in the SNpc (despite inducing  
04 dopaminergic neurodegeneration – (30) suggesting that this phenomenon is specific to seeding  
05 experiment paradigms.

06 Lastly, we analyzed a publicly available cortical proteomic database of healthy individual and PD  
07 patients. Of interest, we observed that several  $Zn^{2+}$  transporters were elevated in the brains of PD  
08 patients thus suggesting a zinc dyshomeostasis in patients (Fig. S7F). Indeed, plasma membrane  
09 transporters such as the zinc transporter 1 (ZnT1), the Zrt-/Irt-like protein 6 (ZIP6) and ZIP10  
10 showed increased levels (Fig. S7G-I) while the synaptic vesicle membrane transporter ZnT3  
11 remained constant (Fig. S7J).

12

### 13 *Association metric shows independence of strong predictors*

14 As we used combinations of 3 variables and because of the structure of MLPs, one could expect  
15 that some combinations would complement each other to allow finer prediction of  
16 neurodegeneration levels. To address this question, we used a classic measurement of association  
17 in the field of data-mining: lift (31) and plotted the results as network plots showing association  
18 (edge size) and enrichment in the best learners (node size). Lift calculation was corrected for error  
19 prediction to avoid detrimental association between variables. The first observation was that the

20 most enriched variables (top 3 to 5) appeared to be self-sufficient to predict the neurodegeneration  
21 levels with minimal error (Fig. 6). Some variables, with modest enrichment, showed strong positive  
22 associations that were specific to each experimental group. Associated variables in LB-injected  
23 monkeys were: (i)  $\alpha$ -syn-related parameters along the SNpc-striatum-cortex axis, an impairment of  
24 locomotion and the ethologically-defined orientation of the animals towards their environment (Fig.  
25 6 top left inset); (ii) oligomeric  $\alpha$ -syn species measured in the midbrain and striatum equally  
26 associated, but to lesser extent, with  $\alpha$ -syn levels in cortex and plasma (Fig. 6 top right inset).  
27 In noLB-injected animals, the analysis shed light upon the relative abundance of two members of  
28 the macroautophagy pathway (Fig. 6B top left) as well as the balance between monomeric and high-  
29 molecular weight species of  $\alpha$ -syn in the putamen (Fig. 6B bottom right). Such disruption of the  
30 nigrostriatal pathway has repercussions upon the basal ganglia physiology as GABA levels in their  
31 output structure, the internal globus pallidus, was associated with a decreased social behavior (Fig.  
32 6B bottom left inset).

33

## 34 **DISCUSSION**

35 In the present study, we report that, in non-human primates, injection of distinct  $\alpha$ -syn assemblies  
36 derived from PD patients lead to dopaminergic degeneration through discrete mechanisms.  
37 Applying a machine-learning method, we gained insight into unique signatures of degeneration  
38 induced by injection of two distinct  $\alpha$ -syn pathogenic assemblies (i.e. those contained in the LB  
39 and noLB fractions derived from idiopathic PD patients' brains). To do so, we built a large dataset  
40 with 180 variables obtained from behavioral, histological, biochemical, transcriptional and  
41 biophysical approaches applied to several brain areas for each individual. By using a distributed  
42 MLP algorithm that we developed for the purpose of this study, we identified characteristics that  
43 give insight into the strongest predictors of neurodegeneration for each experimental group. We  
44 have, therefore, described for the first time that distinct  $\alpha$ -syn assemblies leading to similar  
45 degeneration in monkeys are associated with different mechanisms, hence experimentally  
46 confirming the true multifactorial nature of synucleinopathies.

47 Our results illustrate that both small oligomeric as well as larger  $\alpha$ -syn assemblies induce  
48 dopaminergic degeneration in non-human primates. This finding was unexpected, since previous  
49 mouse studies from our laboratory showed that noLB injection did not have any observable  
50 consequence regarding dopaminergic degeneration,  $\alpha$ -syn accumulation or phosphorylation (11).  
51 In agreement, other groups also showed the absence of toxicity of soluble recombinant  $\alpha$ -syn (12).

52 One possible explanation is that primate dopaminergic neurons could be highly susceptible to  $\alpha$ -  
53 syn toxicity. This could be in part due to their unique cellular architecture (32), a feature already  
54 known to contribute to the selective vulnerability of these neurons in PD (33). In fact, the large and  
55 complex axonal arbor of dopamine neurons make them particularly vulnerable to factors that  
56 contribute to cell death and, in primates, this axonal arbor is ten-fold the size of that in rodents  
57 (32). In addition, primate dopamine neurons display unique molecular characteristics (e.g. the  
58 presence of neuromelanin, the intracellular levels of which have been shown to be important in the  
59 threshold for the initiation of PD) (34). These unique features of primate dopaminergic neurons  
60 might be important in explaining the toxic mechanisms of the relatively low content of  $\alpha$ -syn  
61 aggregates in the noLB fractions. Additional studies are now needed to fully address the question  
62 of host-seed interactions, but our results highlight the relevance and the need of the non-human  
63 primate model for the study of synucleinopathies.

64 We also confirmed that the toxicity mechanisms associated with patient-derived  $\alpha$ -syn aggregates  
65 are shared features among patients and, therefore, common to the disease. Indeed, LB and noLB  
66 fractions used in this study were isolated from a pool of 5 patients who were different from the pool  
67 of 3 patients used in our previous study in mice (11). In the mice experiment (Fig. S3B) performed  
68 in this study, we observed the same level of dopaminergic degeneration (~40% at 4 months after  
69 injection).

70 The surprising observation, in non-human primates, that the noLB fraction is toxic to the same  
71 extent as the LB fraction suggests the existence of previously unrecognized forms of  $\alpha$ -syn toxicity.  
72 Several studies have suggested that pre-fibrillar oligomeric species are the toxic  $\alpha$ -syn species (8,  
73 9). Our biochemical studies showed that noLB and LB fractions had different amyloid properties  
74 (Fig. 1), contents (Fig. S1, S2A) and structures (Fig. S2B-E). Indeed, LB fractions contained a  
75 majority of large aggregated  $\alpha$ -syn fibrils as well as some smaller aggregates while noLB fractions  
76 contained a smaller proportion (10 folds) of smaller aggregates and soluble  $\alpha$ -syn. More  
77 importantly, the smaller aggregates were different in nature between LB and noLB fractions, as  
78 shown by micro-infrared spectroscopy (Fig. S2B-E). One could hypothesize that the observed effect  
79 is due to a species common between LB and noLB. However, because of the extent of degeneration,  
80 which was similar between the two experimental groups, and the  $\alpha$ -syn content dissimilarity, both  
81 in amount and nature, this appears very unlikely. We believe that our results support the notion of  
82 the existence of a range of  $\alpha$ -syn pathogenic structures with distinct toxic properties within the PD  
83 brain. Further work is necessary to provide a complete structural characterization of those species.  
84 As yet, very few studies report the high-resolution structures of  $\alpha$ -syn aggregates, which are on the

85 one hand, only derived from studies using recombinant  $\alpha$ -syn and, on the other hand, limited to  
86 near atomic resolution (35-37). Encouragingly, much effort is currently being devoted to this field  
87 of research and two recent studies reported the atomic structure of  $\alpha$ -syn fibrils determined by cryo-  
88 electron microscopy (38, 39), while still being limited to recombinant-generated  $\alpha$ -syn, and not  
89 isolated from human brain tissue.

90 In order to perform a characterization of the effects of the two fractions, we developed a machine  
91 learning method to identify their biological characteristics. It is now well accepted that machine  
92 learning algorithms can be trained to detect patterns as well as, or even better than, humans (40-  
93 42). Instead of the classification algorithms (the algorithm learns to identify in which category a  
94 sample belongs) that were mostly used in recent applications of machine learning in biology (43),  
95 we chose in this study to predict continuous and biologically-relevant variables using MLPs. Our  
96 choice was motivated by the limited sample size that is often a constraint of experimental biology.  
97 Although it might have been possible to use other feature selection methods, the use of MLPs with  
98 a distributed architecture allowed us to avoid overfitting issues and to develop a method particularly  
99 well-suited for low sample size datasets (44). As both LB and noLB-injected monkeys displayed  
00 similar levels of degeneration, they were indistinguishable using that endpoint. Instead of using a  
01 clustering analysis or a classification method, hence making the *a priori* assumption that these  
02 groups were different, we preferred to submit the two experimental groups to the MLP  
03 independently.

04 The combination of this constrained, distributed architecture and the holistic approach allowed us  
05 to rank input variables according to the number of times they appeared in the group of best  
06 predictors (defined as top 1% of best networks). A major issue in the use of machine learning in  
07 experimental biology in the 'black-box' is the fact that it is usually impossible to 'understand' how  
08 an algorithm predicted an output (45). By using a reverse engineering method, we aimed to tackle  
09 that issue. Because we explored all possible combinations of our variables, we could rank the input  
10 variables assuming that the more they appeared in the top 1%, the more they contained information  
11 allowing precise prediction of the neurodegeneration levels. Interestingly, our two experimental  
12 groups showed that some of the best predictors were similar (about 30%) but the majority were  
13 different. One could hypothesize that the similar variables between the two signatures probably  
14 embedded information that are consequences of neurodegeneration while the different ones  
15 probably contain information regarding the process of disease initiation and/or progression. Further  
16 experimental studies are now needed to confirm the relevance of these variables.

17 Also, as these two kinds of  $\alpha$ -syn assemblies were associated with different signatures identified  
18 by our MLP approach, we propose that our results illustrate the multifactorial nature of the disease  
19 as different mechanisms (i.e. signatures) initiated by different triggers (i.e.  $\alpha$ -syn assemblies) led  
20 to similar consequences (i.e. degeneration levels).

21 Using this methodology, we confirmed the interest of highly-expected variables but more  
22 importantly also unexpected variables that appear to be excellent predictors of  $\alpha$ -syn-associated  
23 dopaminergic degeneration. The first hit for LB-injected animals was phosphorylated  $\alpha$ -syn in the  
24 entorhinal cortex (as we have previously shown) followed by phosphorylated  $\alpha$ -syn in the para-  
25 hippocampal cortex (unexpected), striatal microglial activation and GABA dysregulation in the  
26 internal part of the globus pallidus (expected) (Fig. S5). Conversely, Zn homeostasis was a strong  
27 predictive variable (unexpected) followed by  $\alpha$ -syn aggregation-related terms (expected) in noLB-  
28 injected animals (Fig. S6).

29 In order to confirm the prediction made by the MLP approach, we first performed a retrospective  
30 literature analysis. This analysis showed that a significant part of the best predictors has been shown  
31 in the literature to be correlated with disease progression. Then, we attempted to confirm the interest  
32 of one of the top hits, the accumulation of  $Zn^{2+}$  in the SNpc, in independent experimental cohorts.  
33 Interestingly, we here describe that both in mice injected with noLB or in macaque monkeys (a  
34 different non-human primate sub species that the baboons used in that study) injected either in the  
35 striatum or in the SNpc, Zn levels were increased in the SNpc. However, in mice, Zn  
36 dyshomeostasis was not associated with neurodegeneration in the noLB group (at odds with what  
37 was observed in monkeys) suggesting a species difference in the relationship between zinc levels  
38 and dopaminergic tract integrity. Surprisingly, that result was not observed in rats and marmoset  
39 monkeys overexpressing human mutant  $\alpha$ -syn. This observation might suggest that Zn  
40 dyshomeostasis is a feature of disease not triggered in the context of human mutant  $\alpha$ -syn  
41 overexpression that is associated with fast progressing pathology (Bourdenx et al. 2015). Then, in  
42 order to expand our results to human pathology, we analyzed a publicly available proteomic dataset  
43 of human samples. According to that analysis, PD patients displayed increased levels of plasma  
44 membrane Zn transporters, hence suggesting a Zn dyshomeostasis in patients. In the context of PD,  
45 Zn dyshomeostasis has been associated with autophagy/lysosomal dysfunction in the context of  
46 *PARK9* mutations (Ramirez et al. 2006, Dehay et al. 2012). Further studies are now needed to fully  
47 unravel this connection.

48 Altogether, our findings show that primate dopaminergic neurons are sensitive to both small, mostly  
49 soluble,  $\alpha$ -syn extracts as well as larger, aggregated,  $\alpha$ -syn extracts derived from PD patients. These

50 findings involve two immediate outcomes. First, since this toxicity has not been reported so far it  
51 suggest species differences that would need to be thoroughly investigated (46, 47) and calls for a  
52 systematic appraisal of proteinopathies in primates in particular for validating therapeutic strategies  
53 before clinical testing (48). Second, the present study highlights the complex structure-toxicity  
54 relationship of  $\alpha$ -syn assemblies and corroborates the multifactorial origin of synucleinopathies as  
55 distinct assemblies can induce similar degeneration (that would probably lead to similar clinical  
56 manifestation in patients) through different mechanisms, nigrostriatal or extranigral brain  
57 pathways, calling for molecular diagnosis to identify patient sub-populations before launching  
58 large-scale, heterogeneous in nature, clinical trials. Finally, we developed a machine-learning  
59 approach allowing and quantitative assessment of the explanatory power of a given set of variables  
60 compatible with the constrained sample size of experimental biology.

61

62



## 63 MATERIALS AND METHODS

### 64 Access to data and machine-learning code for replicability and further use by the community

65 The entire raw data set is made available to the readers (Table S2). Authors chose not to provide  
66 representative examples of each procedure for the sake of space and because the entire data set is  
67 fully disclosed. Further information and requests for examples should be directed to and will be  
68 fulfilled by the Corresponding Contacts. Hyperlink to the machine-learning code  
69 (10.5281/zenodo.1240558) is provided (<https://zenodo.org/record/1240558#.XC8pqy17Su4>).

### 71 Ethics statement

72 Experiments were performed in accordance with the European Union directive of September 22,  
73 2010 (2010/63/EU) on the protection of animals used for scientific purposes. The Animal  
74 Experimentation Ethical Committee (CEEA) of the Vall d'Hebron Institute of Research (VHIR)  
75 approved experiments under the license number CEEA 81/13 (rats). The Institutional Animal Care  
76 and Ethical Committee of Bordeaux University (CE50, France) approved experiments under the  
77 license number 5012099-A (mice). The Institutional Animal Care and Ethical Committee of Murcia  
78 University (Spain) approved experiments under the license number REGA ES300305440012  
79 (monkeys).

### 81 Animals and Stereotactic Injections

82 *Mice.* Wild-type C57BL/6 mice (4 months old) received 2 $\mu$ l of either LB fractions or noLB  
83 fractions by stereotactic delivery to the region immediately above the right substantia nigra  
84 (coordinates from Bregma: AP=-2.9, L= -1,3, DV=-4.5) at a flow rate of 0.4 $\mu$ l/min and the pipette  
85 was left in place for 5 min after injection to avoid leakage. Mice were killed four months after  
86 injection. Ten to fifteen mice were used in each group.

87 *Monkeys.* Animals, which were from the research animal facility of the University of Murcia  
88 (Murcia, Spain) and housed in 2 multi-male multi-female exterior pens, were studied in a breeding  
89 farm over 2 years (Murcia, Spain). Animals were fed fruits, vegetables and monkey pellets twice a  
90 day before 9 am and after 5pm. Water was available ad libitum. 17 healthy adult olive baboons  
91 (*Papio papio*) were used in this study. Group sizes were chosen assuming a one-tailed alpha of 0.05,  
92 with sample size of at least three per group, which provided >80% power to detect a difference  
93 between the treatment groups and the control group, using a Fisher's exact test. Animals were  
94 randomized into treatment or control groups. Six baboons were used for LB injections, four were  
95 used for noLB injections and seven were untreated control animals. Intrastratial injections of either

96 LB fractions or noLB fractions were performed at 2 rostrocaudal levels of the motor striatum  
97 (anterior commissure [AC], -1mm and -5mm) under stereotactic guidance as previously described  
98 (49-52). The total injected volume per hemisphere was 100 $\mu$ l (2 injection sites with 50 $\mu$ l each at  
99 3 $\mu$ l/min at each location site). After each injection, the syringe was left in place for 10 min to  
00 prevent leakage along the needle track. A number of parameters were monitored during the course  
01 of the two-year study, including survival and clinical observations. At the end of the experiment  
02 (24 months post-injection), all monkeys were euthanised with pentobarbital overdose (150mg/kg  
03 i.v.), followed by perfusion with room-temperature 0.9% saline solution (containing 1% heparin)  
04 in accordance with accepted European Veterinary Medical Association guidelines. Brains were  
05 removed quickly after death. Each brain was then dissected along the midline and each hemisphere  
06 was divided into three parts. The left hemisphere was immediately frozen by immersion in  
07 isopentane at -50°C for at least 5 min and stored at -80°C. The right hemisphere was fixed for one  
08 week in 10 vol/tissue of 4% paraformaldehyde at 4°C, cryoprotected in two successive gradients of  
09 20 then 30% sucrose in phosphate buffered saline (PBS) before being frozen by immersion in  
10 isopentane (-50°C) for at least 5 min and stored at -80°C until sectioning. CSF and blood samples  
11 (plasma, serum, whole blood) in the 17 animals were carefully collected before euthanasia. No  
12 samples were excluded from analysis in these studies.

13

#### 14 **Purification of Lewy bodies from human PD Brains**

15 The samples were obtained from brains collected in a Brain Donation Program of the Brain Bank  
16 “GIE NeuroCEB” run by a consortium of Patients Associations: ARSEP (association for research  
17 on multiple sclerosis), CSC (cerebellar ataxias), France Alzheimer and France Parkinson. The  
18 consents were signed by the patients themselves or their next of kin in their name, in accordance  
19 with the French Bioethical Laws. The Brain Bank GIE NeuroCEB (Bioresource Research Impact  
20 Factor number BB-0033-00011) has been declared at the Ministry of Higher Education and  
21 Research and has received approval to distribute samples (agreement AC-2013-1887). Human  
22 SNpc was dissected from fresh frozen postmortem midbrain samples from 5 patients with sporadic  
23 PD exhibiting conspicuous nigral LB pathology on neuropathological examination (mean age at  
24 death:  $75 \pm 2.75$  years; frozen post-mortem interval:  $31.8 \pm 7.45$ h; GIE Neuro-CEB BB-0033-  
25 00011). Tissue was homogenized in 9 vol (w/v) ice-cold MSE buffer (10 mM MOPS/KOH, pH 7.4,  
26 1M sucrose, 1mM EGTA, and 1mMEDTA) with protease inhibitor cocktail (Complete Mini;  
27 Boehringer Mannheim) with 12 strokes of a motor-driven glass/teflon dounce homogenizer. For  
28 LB purification, a sucrose step gradient was prepared by overlaying 2.2 M with 1.4 M and finally  
29 with 1.2 M sucrose in volume ratios of 3.5:8:8 (v/v). The homogenate was layered on the gradient

30 and centrifuged at 160,000 x g for 3 h using a SW32.1 rotor (Beckman). Twenty-six fractions of  
31 1500  $\mu$ l were collected from each gradient from top (fraction 1) to bottom (fraction 26) and analyzed  
32 for the presence of  $\alpha$ -synuclein aggregates by filter retardation assay, as previously described (11).  
33 Further characterization of LB fractions was performed by immunofluorescence,  $\alpha$ -synuclein  
34 ELISA quantification and electron microscopy as previously described (11). For stereotactic  
35 injections, LB-containing fractions from PD patients were mixed together in the same proportion  
36 (PD#1, fractions 19 and 20; PD#2, fractions 19 and 20; PD#3, fraction 22; PD#4, fractions 17,18  
37 and 19; PD#5, fractions 20, 21 and 23). NoLB-containing fractions (i.e. fraction 3, at the beginning  
38 of the 1,2M interface) derived from the same PD patients (which contain soluble or finely granular  
39  $\alpha$ -synuclein) but lacks large LB-linked  $\alpha$ -synuclein aggregates were obtained from the same sucrose  
40 gradient purification. Using enzyme-linked immunosorbent assay (ELISA) kit against human  $\alpha$ -  
41 synuclein (Invitrogen, #KHB0061 – following manufacturer's recommendations),  $\alpha$ -syn  
42 concentration was measured and both LB and noLB fractions were adjusted to ~24 pg  $\alpha$ -synuclein  
43 per microliter. In all cases, samples were bath-sonicated for 5 min prior to *in vitro* and *in vivo*  
44 injections.

#### 45 46 **Characterization of noLB and LB fractions**

47 *Electron microscopy.* Briefly, carbon-coated nickel grids were covered for 1 min with  
48 corresponding fractions of interest, then washed 3 times with distilled water. They were then  
49 washed again in distilled water and stained for 5 min with 2% uranyl acetate, before being air-dried.  
50 Digital images were obtained with a computer linked directly to a CCD camera (Gatan) on a Hitachi  
51 H-7650 electron microscope. In all cases, samples were bath-sonicated for 5 min prior to the *in*  
52 *vitro* applications.

53 *Immunofluorescence analysis of noLB and LB fractions.* Indicated fractions from the sucrose  
54 gradient were spread over slides coated with poly-D lysine and fixed with 4% paraformaldehyde  
55 (PFA) in PBS for 30 min. Fixed slides were stained with 0.05% thioflavin S for 8 min and then  
56 washed three times with 80% EtOH for 5 min, followed by two washes in PBS for 5 min. Finally,  
57 all samples were washed 3 times with PBS and blocked with 2% casein and 2% normal goat serum  
58 for 30 min. For immunofluorescence analyses, samples were incubated with human  $\alpha$ -synuclein  
59 specific antibody (clone syn211, Thermo Scientific, 1:1000) for 30 min, washed three times with  
60 PBS, incubated with a goat anti-mouse TRITC (Jackson, 1:500), before being cover-slipped for  
61 microscopic visualization using fluorescence mounting medium.

62 *Dot-blotting analysis.* To evaluate PK-resistant  $\alpha$ -synuclein contained in noLB and LB fractions  
63 derived from PD brains, each fraction was subjected to digestion with 1  $\mu$ g/ml proteinase K for 0,

64 15, 30, 45, and 60 min. The reaction was stopped by boiling for 5 min before dot-blotting with  
65 syn211 antibody. To analyze their stability, noLB and LB fractions were treated with increasing  
66 concentrations of urea (7 and 8M) or sodium dodecyl sulfate (SDS) (0.5, 1 and 2%) for 6 h at room  
67 temperature.  $\alpha$ -Synuclein was visualized as described above.

68 Filter retardation assay of noLB and LB fractions were probed with antibodies against,  
69 phosphorylated  $\alpha$ -synuclein (Abcam EP1536Y, 1:1000), ubiquitin (Sigma-Aldrich U5379, 1:1000),  
70 p62 (Progen GR62-C, 1:1000), hyperphosphorylated tau (AT8, MN1020, ThermoFischer) or A $\beta$   
71 (DAKO clone 6F/3D, 1:1000).

72 *Human  $\alpha$ -Synuclein aggregation TR-FRET immunoassay.* Time-resolved Förster's resonance  
73 energy transfer (TR-FRET)-based immunoassays were validated for total and oligomeric  $\alpha$ -  
74 synuclein (53). Ten microliters of noLB and LB samples were analyzed for total  $\alpha$ -synuclein  
75 quantification with the TR-FRET immunoassays kit against human  $\alpha$ -synuclein aggregation kit  
76 (Cisbio, #6FASYPEG) according to the manufacturer's instructions.

77 *Velocity sedimentation and density floatation  $\alpha$ -synuclein profiles in noLB and LB fractions.* Frozen  
78 noLB and LB fractions aliquots (100  $\mu$ L) were thawed and solubilized in solubilization buffer (SB)  
79 to reach 10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, Complete EDTA-free  
80 protease inhibitors (Roche), PhosSTOP phosphatase inhibitors (Roche), 1 U/ $\mu$ L Benzonase  
81 (Novagen), 2 mM MgCl<sub>2</sub> and 2% (w/v) N-lauroyl-sarcosine (sarkosyl, Sigma) final concentrations,  
82 by incubating at 37°C under constant shaking at 600 rpm (Thermomixer, Eppendorf) for 45 minutes.  
83 For velocity sedimentations, a volume of 400  $\mu$ L of solubilized noLB / LB fraction was loaded on  
84 top of a 11 mL continuous 5-20% iodixanol gradient (Optiprep, Sigma) in SB buffer containing  
85 0.5% w/v final sarkosyl concentration, linearized directly in ultracentrifuge 11 mL tubes (Seton)  
86 with a Gradient Master (Biocomp). For density floatation gradients, a volume of 400  $\mu$ L of  
87 solubilized noLB / LB fraction was mixed to reach 40% iodixanol in SB buffer with 0.5% w/v final  
88 sarkosyl concentration and loaded within an 11 mL 10-60% discontinuous iodixanol gradient in SB  
89 buffer with 0.5% w/v final sarkosyl concentration. The gradients were centrifuged at 180,000 g for  
90 3 hours (velocity) or for 17 hours (density) in a swinging-bucket SW-40 Ti rotor using an Optima  
91 L-90K ultracentrifuge (Beckman Coulter). Gradients were then segregated into 16 equal fractions  
92 from the top using a piston fractionator (Biocomp) and a fraction collector (Gilson). Fractions were  
93 aliquoted for further analysis of their content by dot-blot. Gradient linearity was verified by  
94 refractometry.

95 For dot blotting, aliquots of the collected native fractions were spotted onto Hybond PVDF 0.2  $\mu$ m  
96 membranes (GE Healthcare) using a dot blot vacuum device (Whatman). For total (MJFR1) and  
97 phosphorylated pS129 (EP1536Y)  $\alpha$ -synuclein immunolabelling, a step of fixation in PBS - 0.1%

98 glutaraldehyde was performed at this point, followed by 3 washes in PBS. Membranes were then  
99 blocked with 5 % (w/v) skimmed milk powder in PBS - 0.1% (v/v) Tween and probed with anti-  
00 human  $\alpha$ -synuclein (MJFR1, rabbit 1:10000, Abcam), anti-phospho pS129  $\alpha$ -synuclein (EP1536Y,  
01 rabbit 1:5000, Abcam) or anti  $\alpha$ -synuclein aggregate specific FILA-1 (MJFR14-6-4-2, rabbit  
02 1:10000, Abcam) primary antibodies in PBS-T - 4% (w/v) BSA, and secondary goat anti rabbit IgG  
03 HRP-conjugated antibodies (1:10000, Jackson Laboratories) in PBS-T 1% (w/v) milk.  
04 Immunoreactivity was visualized by chemiluminescence (GE Healthcare). The amount of the  
05 respective protein in each fraction was determined by the Image Studio Lite software, after  
06 acquisition of chemiluminescent signals with a Chemidoc imager (Biorad). Profiles obtained by  
07 immunoblot were normalized and plotted with SEM using the Prism software.

08 *FTIR microspectroscopy.* 1-2  $\mu$ L of each suspension was deposited on a CaF<sub>2</sub> window and dried at  
09 room pressure and temperature. The protein aggregates were then measured in transmission at  
10 50x50  $\mu$ m<sup>2</sup> spatial resolution with an infrared microscope (54). Depending on its size it was  
11 possible to collect one to twenty spectra inside each aggregate. The infrared microscope was a  
12 Thermo Scientific Continuum equipped with a MCT detector and a 32x 0.65 NA Replachromat  
13 objective and matching condenser, coupled to a Thermo Scientific Nicolet 8700 spectrometer with  
14 a globar source and KBr beamsplitter. The microscope was operated in dual path single aperture  
15 mode. Spectra were recorded between 650-4000  $\text{cm}^{-1}$  at 2  $\text{cm}^{-1}$  resolution, with Happ-Genzel  
16 apodization and Mertz phase correction. Spectra were processed in Omnic 9.2 for automatic  
17 atmospheric correction to remove water vapor contribution.

18

### 19 **Rat Ventral Midbrain Primary Cultures**

20 Postnatally derived ventral midbrain cultures were prepared essentially as previously described  
21 (55). Briefly, cultures were prepared in two steps. In the first step, rat astrocyte monolayers were  
22 generated as follows. The entire cerebral cortex from a single rat pup (postnatal days 1–2) was  
23 removed, diced, and then mechanically dissociated by gentle trituration. The isolated cells were  
24 plated at 80,000 cells per well under which a laminin-coated coverslip was affixed. The cells were  
25 housed at 37°C in an incubator in 5% CO<sub>2</sub> and were fed on glial media (89% MEM, 9.9% calf  
26 serum, 0.33% glucose, 0.5 mM glutamine, and 5  $\mu$ g/mL insulin). Once confluence had been attained  
27 (about 1 week in vitro), fluorodeoxyuridine (6.7 mg/mL) and uridine (16.5 mg/mL) were added to  
28 prevent additional proliferation. In the second stage, which occurred 1 week later, rat pups aged  
29 between 1 and 2 days were anesthetized and 1-mm<sup>3</sup> blocks containing ventral midbrain neurons  
30 were dissected from 1-mm-thick sagittal sections taken along the midline of the brain. Tissues were  
31 collected immediately into cold phosphate buffer and were treated enzymatically using papain (20

32 U/mL) with kynurenate (500  $\mu$ M) at 37°C under continuous oxygenation with gentle agitation for  
33 2 h. A dissociated cell suspension was achieved by gentle trituration and was then plated onto the  
34 preestablished glia wells at a density of 0.5–1.7 million neurons per well. Cultures were maintained  
35 in specially designed neuronal media (47% MEM, 40% DMEM, 10% Hams F-12 nutrient medium,  
36 1% calf serum, 0.25% albumin, 2 mg/mL glucose, 0.4 mM glutamine, 10  $\mu$ g/mL catalase, 50  $\mu$ M  
37 kynurenic acid, 10  $\mu$ M CNQX, 25  $\mu$ g/mL insulin, 100  $\mu$ g/mL transferrin, 5  $\mu$ g/mL superoxide  
38 dismutase, 2.4  $\mu$ g/mL putrescine, 5.2 ng/mL Na<sub>2</sub>SeO<sub>3</sub>, 0.02  $\mu$ g/mL triiodothyronine, 62.5 ng/mL  
39 progesterone, and 40 ng/mL cortisol) containing 27  $\mu$ M fluorodeoxyuridine and 68  $\mu$ M uridine to  
40 control glial outgrowth and in 10 ng/mL glial cell derived neurotrophic factor (GDNF). They were  
41 incubated for a further 7–8 days until the start of experiments. All tyrosine hydroxylase (TH)  
42 neurons were counted on each plate following the addition of noLB and LB fractions after 1, 2, 5  
43 and 7 days of treatment.

44

#### 45 **Non-Human Primate Behavioral Assessment**

46 Following a 4-hour minimum habituation phase performed one day before the beginning of the  
47 observations, baboon behavior was observed outside the feeding and cleaning times, in a random  
48 order at two-time points (morning and afternoon), over 4 to 9 days (8 sessions per group). On the  
49 1st observational time point (i.e. 1-month post-surgery), the habituation phase was performed over  
50 3 days allowing the observer to recognize the animals individually. We used a scan-sampling  
51 method, appropriate for time budgeting (56), in which behavioral parameters were assessed every  
52 5 minutes during 2-hour sessions, resulting in 192 scans per individual. Extra observational sessions  
53 were performed to avoid missing data. A unique trained observer (SC; intra-observer reliability:  
54 Spearman rank order correlation R=0.987) collected the data live on the 2-time points of the study:  
55 at 1- and 24-months post-surgery. The observer was standing 1 m away from the outdoor cages.  
56 We focused on behavioral profiles rather than single items and used two repertoires: one reports  
57 the interaction with the environment and one describes the position within the environment,  
58 according to published protocols (57-59). We investigated the percentages of occurrence of each  
59 item with regard to the total number of scans in order to obtain mean behavioral and postural time  
60 budgets, body orientation and location profiles.

61

#### 62 **Histopathological analysis**

63 *Extent of lesion.* To assess the integrity of the nigrostriatal pathway, tyrosine hydroxylase (TH)  
64 immunohistochemistry was performed on SNpc and striatal sections. Briefly, 50 $\mu$ m free-floating  
65 sections from one representative level of the striatum (anterior, medial and posterior) and serial

66 sections (1/12) corresponding to the whole SNpc were incubated with a mouse monoclonal  
67 antibody raised against human TH (Millipore, MAB318, 1:5000) for one night at RT and revealed  
68 by an anti-mouse peroxidase EnVision™ system (DAKO, K400311) followed by DAB  
69 visualization. Free-floating SNpc sections were mounted on gelatinized slides, counterstained with  
70 0.1% cresyl violet solution, dehydrated and coverslipped, while striatal sections were mounted on  
71 gelatinized slides and coverslipped. The extent of the lesion in the striatum was quantified by optical  
72 density (OD). Sections were scanned in an Epson expression 10000XL high resolution scanner and  
73 images were used in ImageJ open source software to compare the grey level in each region of  
74 interest: i.e. caudate nucleus and putamen. TH-positive SNpc cells were counted by stereology blind  
75 with regard to the experimental condition using a Leica DM6000B motorized microscope coupled  
76 with the Mercator software (ExploraNova, France). The substantia nigra was delineated for each  
77 slide and probes for stereological counting were applied to the map obtained (size of probes was  
78 100x80µm spaced by 600x400µm). Each TH-positive cell with its nucleus included in the probe  
79 was counted. The optical fractionator method was finally used to estimate the total number of TH-  
80 positive cells in the SNpc of each monkey hemisphere. In addition, we measured Nissl cell count,  
81 the volume of SN, and the surface of TH-occupied in SN to fully characterize the pattern of  
82 dopaminergic cell loss in the SN.

83 *α-synuclein pathology.* Synucleinopathy was assessed with a mouse monoclonal antibody raised  
84 against human α-synuclein (syn211) and phosphorylated α-synuclein (clone11A5, Elan, 1:5000)  
85 immunostaining as we previously reported (11, 30). Briefly, selected sections at two rostro-caudal  
86 levels were incubated in a same well to allow direct comparison of immunostaining intensity.  
87 Sections were incubated overnight at room temperature with the aforementioned antibodies. The  
88 following day, revelation was performed with anti-specie peroxidase EnVision system (DAKO)  
89 followed by 3,3' -diaminobenzidine (DAB) incubation. Sections were then mounted on gelatinized  
90 slides, dehydrated, counterstained if necessary and coverslipped until further analysis. Grey level  
91 quantification or immunostaining-positive surface quantification in forty brain regions (Fig. 2B)  
92 were performed as previously described (30).

93 *Inflammation.* Inflammatory process in the striatum, in the entorhinal cortex and in the white matter  
94 of noLB and LB-injected monkeys was measured through GFAP/S-100 (DAKO, Z0334/Abnova,  
95 PAP11341) and Iba1 (Abcam, ab5076) immunohistochemistry. Striatal sections of all animals were  
96 incubated together over night with a mix of rabbit antibodies raised against GFAP and S-100 for  
97 the astroglial staining (respective dilutions 1:2000 and 1:1000) and with a goat anti-Iba1 antibody  
98 for the microglial staining (dilution 1:1000). These signals were revealed with anti-specie peroxidase  
99 EnVision system (DAKO) followed by DAB incubation. Sections were mounted on slides, counter-

00 stained in 0.1% cresyl violet solution, dehydrated and cover-slipped. Sections stained by GFAP-S-  
01 100 were numerized at x20 magnification with a NanoZoomer (Hamamatsu, France) and the  
02 quantification of GFAP-positive astrocytic reaction was estimated by a immunostaining-positive  
03 surface quantification at regional levels with the Mercator software (ExploraNova, France).  
04 Sections stained by Iba1 were used for the microglial morphology analysis through fractal  
05 dimension quantification based on microscopic acquisitions, as previously described(60). All  
06 analyses were performed blinded to the researcher.

07

### 08 **mRNA extraction and qRT-PCR**

09 Substantia nigra samples were homogenized in Tri-reagent (Euromedex, France) and RNA was  
10 isolated using a standard chloroform/isopropanol protocol(61). RNA was processed and analyzed  
11 following an adaptation of published methods(62). cDNA was synthesized from 2 µg of total RNA  
12 using RevertAid Premium Reverse Transcriptase (Fermentas) and primed with oligo-dT primers  
13 (Fermentas) and random primers (Fermentas). QPCR was performed using a LightCycler® 480  
14 Real-Time PCR System (Roche, Meylan, France). QPCR reactions were done in duplicate for each  
15 sample, using transcript-specific primers, cDNA (4 ng) and LightCycler 480 SYBR Green I Master  
16 (Roche) in a final volume of 10 µl. The PCR data were exported and analyzed in an informatics  
17 tool (Gene Expression Analysis Software Environment) developed at the NeuroCentre Magendie.  
18 For the determination of the reference gene, the Genorm method was used(63). Relative expression  
19 analysis was corrected for PCR efficiency and normalized against two reference genes. The  
20 proteasome subunit, beta type, 6 (Psm6) and eukaryotic translation initiation factor 4a2 (EIF4A2)  
21 genes were used as reference genes. The relative level of expression was calculated using the  
22 comparative ( $2^{-\Delta\Delta CT}$ ) method(63).

23 Primers sequences: Psm6 (NM\_002798) forward: CAAGAAGGAGGGCAGGTGTACT; Psm6  
24 (NM\_002798) reverse: CCTCCAATGGCAAAGGACTG; EIF4a2 (NM\_001967) forward:  
25 TGACATGGACCAGAAGGAGAGA; EIF4a2 (NM\_001967) reverse:  
26 TGATCAGAACACGACTTGACCCT; SNCA (CR457058) forward: GGGCAAGAATGAA  
27 GAAGGAGC; SNCA (CR457058) reverse: GCCTCATTGTCAGGATCCACA.

28

### 29 **Biochemical analysis**

30 *Total protein extraction and quantification.* Immunoblot analyses were performed on substantia  
31 nigra, putamen and caudate nucleus. Five tissue patches were extracted on ice using 100µl of RIPA  
32 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.5% Na-deoxycholate, 0.1%  
33 sodium dodecyl sulfate) with a protease inhibitor cocktail tablet (Complete Mini, Roche



34 Diagnostics). The lysate was placed on ice for 20 min and then centrifuged at 14,000rpm for 15  
35 min at 4°C. The supernatant was collected and the Bicinchoninic Acid (BCA) Assay was used to  
36 determine the total amount of protein in the lysates, and then stored at -80°C.

37 Based on total protein concentrations calculated from the BCA assays, aliquots of tissue lysates  
38 corresponding to known amounts of total protein per lane were prepared for each animal in Laemmli  
39 buffer (Tris-HCl 25mM pH=6.8, Glycerol 7.5%, SDS 1%, DTT 250mM and Bromophenol Blue  
40 0.05%) for immunoblotting experiment.

41 *Biochemical fractionation.* This technique was performed as described(64). Tissue patches (n=10)  
42 were homogenized in 200µl of high-salt (HS) buffer (50 mmol/L of Tris, 750 mmol/L of NaCl, 5  
43 mmol/L of EDTA, and a cocktail of protease inhibitors and phosphatase inhibitors). Samples were  
44 sedimented at 100,000 × g for 20 minutes, and supernatants were removed for analysis. Pellets were  
45 rehomogenized in successive buffers, after which each was sedimented, and supernatant was  
46 removed: HS containing 1% Triton X-100 (HS/Triton) (Variable names terminated as ultra.s1),  
47 RIPA (50 mmol/L of Tris, 150 mmol/L of NaCl, 5 mmol/L of EDTA, 1% NP40, 0.5% Na  
48 deoxycholate, and 0.1% SDS) (Variable names terminated as ultra.s12, and SDS/urea (8 mol/L of  
49 urea, 2% SDS, 10 mmol/L of Tris; pH 7.5) (Variable names terminated as ultra.p2). Sodium dodecyl  
50 sulfate sample buffer was added, and samples were heated to 100°C for 5 minutes prior to  
51 immunoblot analysis.

52 *Western blot analysis.* Western blots were run in all conditions from 20µg of protein separated by  
53 SDS-PAGE and transferred to nitrocellulose. Incubation of the primary antibodies was performed  
54 overnight at 4°C with rabbit anti-LC3 (1:1000, Novus Biologicals), rabbit anti- LAMP-2 (1:1000,  
55 Santa Cruz Biotechnology), mouse anti-TH (1:1000, Millipore), goat p62 (1:1000, Progen), mouse  
56 anti human- $\alpha$ -synuclein (1:1000, Thermo Scientific). For detection of ubiquitinated proteins,  
57 proteins were transferred on polyvinylidene fluoride membranes (Millipore) and subjected to  
58 Western blot analysis using a rabbit anti-Ubiquitin (1:1000, Sigma U5379). Anti-actin (1:5000,  
59 Sigma) was used to control equal loading. Appropriate secondary antibodies coupled to peroxidase  
60 were revealed using a Super Signal West Pico Chemiluminescent kit (Immobilon Western,  
61 Chemiluminescent HRP substrate, Millipore). Chemiluminescence images were acquired using the  
62 ChemiDoc+XRS system measurement (BioRad). Signals per lane were quantified using ImageJ  
63 and a ratio of signal on loading per animal was performed and used in statistical analyses.

64 *Dot-blot analysis of  $\alpha$ -synuclein.* This technique was performed as we previously described(9, 11).  
65 After heating at 100 °C for 5 min, 20 µg of protein extract was diluted in buffer (25 mM Tris-HCl,  
66 200 mM Glycine, 1% SDS) and filtered through either a nitrocellulose membrane or an acetate  
67 cellulose membrane (Bio-Rad, 0.2 µm pore size). Membranes were then saturated in 5% dry-

68 skimmed milk in PBS and probed with antibodies against  $\alpha$ -synuclein (syn211, 1:1000), both  $\alpha$ -  
69 synuclein fibrils and  $\alpha$ -synuclein oligomers (Syn-O1, 1:10000(65, 66)) (kindly provided by Prof.  
70 Omar El-Agnaf). Revelation was done as described in the previous Materials and Methods section.

71

## 72 **Synchrotron radiation X-ray fluorescence (SR-XRF) microscopy elemental mapping of brain** 73 **tissue cryosections**

74 The synchrotron experiments were carried out at Diamond Light Source, Harwell Science and  
75 Innovation Campus (Didcot, UK) with a 3 GeV energy of the storage ring and 300 mA currents  
76 with top-up injection mode. All SR-XRF microscopy investigations reported herein were carried  
77 out on the microfocus spectroscopy beamline (I18)(67). The micro X-ray fluorescence ( $\mu$ -XRF)  
78 elemental mapping were acquired at room temperature with an incident X-ray energy set to 12 keV  
79 using an Si(111) monochromator and resulting in a X-ray photon flux of  $2 \cdot 10^{11}$  ph/s. The substantia  
80 nigra of each animal were collected from free-floating sections and mounted onto an X-ray  
81 transparent metal-free 4  $\mu$ m thickness Ultralene® foil (SPEXCert Prep, Metuchen, NJ, U.S.A.)  
82 secured to a customized Polyetheretherketone (PEEK) holder ensuring contamination-free samples  
83 and reduced X-ray scattering contribution. The samples were affixed to a magnetic plate that  
84 connects to the sample stage. The 4-element Si drift Vortex ME4 energy dispersive detector  
85 (Hitachi Hi-Technologies Science America) with Xspress-3 processing electronics, was operated  
86 in the 90° geometry, as such it minimizes the background signal. The sample-detector distance was  
87 fixed (75 mm). The sample was held at 45° to the incident X-ray beam and rastered in front of the  
88 beam whilst the X-ray fluorescence spectra were collected. An area of 500  $\mu$ m x 500  $\mu$ m within the  
89 substantia nigra pars compacta (SNpc) was mapped for each sample with a step-size that match the  
90 beam size (5  $\mu$ m) and a dwell time of 1 s per pixel due to low concentration of the element. A thin  
91 (100  $\mu$ m) pellet of the NIST standards reference materials SRM1577c (bovine liver material, NIST,  
92 Gaithersburg, MD, USA) was measured to calibrate experimental parameters as well as a thin-film  
93 XRF reference material (AXO Dresden GmbH). This was followed by elemental quantification  
94 through the open-source software PyMCA(68) in which both the reference material and the sample  
95 are modelled in terms of main composition, density and thickness. The fluorescence spectrum  
96 obtained from each pixel was fitted, the elemental concentration ( $\mu$ g/g dry weight or ppm) maps  
97 were generated and an average elemental concentration of the SNpc regions was obtained.

98

## 99 **Measurement of $\alpha$ -synuclein in monkey biological fluids samples**

00 Multi-Array 96-well plates (MesoScale Discovery, Gaithersburg, MD, USA) were coated with 30 $\mu$ l  
01 3 $\mu$ l/ml MJFR1 (abcam, Cambridge, UK) as capture antibody and incubated overnight at 4°C

02 without shaking. The next day plates were washed 3 times with 150µl PBS-T [PBS (AppliChem,  
03 Darmstadt, Germany) supplemented with 0,05% Tween-20 (Roth, Karlsruhe, Germany)] per well.  
04 Unspecific binding of proteins was prevented by incubation with 150µl 1% BSA (SeraCare Life  
05 Sciences, Milford, MA, USA)/PBS-T/well for 1 hour and shaking at 700rpm. Calibrators (kindly  
06 provided by Prof. Omar El-Agnaf) were prepared from single use aliquots of  $\alpha$ -synuclein (1µg/ml  
07 stored at -80°C until use) and ranged from 25000pg/ml to 6,1pg/ml in serial fourfold dilutions. 1%  
08 BSA/PBS-T served as blank. For the different specimen the following dilutions were applied: 1 in  
09 10000 for whole blood and 1 in 8 for serum, plasma and CSF. All dilutions were prepared in 1%  
10 BSA/PBS-T. After washing the plates 25µl calibrator solutions and diluted samples were applied  
11 to the wells and incubated as indicated above. Plates were washed again and 25µl Sulfo-TAG  
12 labeled Syn1 antibody (BD Biosciences, Heidelberg, Germany) diluted to 1µg/ml in 1% PBS-T  
13 were applied to the wells as detection antibody. Sulfo-TAG labeling was done according to the  
14 manufacturer's instruction using MSD Sulfo-TAG NHS-Ester (MSD). Incubation was for 1 hour  
15 at 700rpm. Plates were washed, 150µl 2x Read Buffer (MSD) was applied and the plates were read  
16 on a MSD SectorImager 2400. Data analysis was performed using WorkBench software (MSD).

### 19 **Neurotransmitter analysis**

20 Brain patches were dissected out on ice-cold plate, weighed and put into 1.5 ml Eppendorf tubes.  
21 Samples were homogenized in methanol/water (50:50% v/v), then centrifuged at 14000 rpm for 15  
22 min at 4°C(69). The supernatant was aliquoted and stored at -80°C until amino acid derivatization.  
23 Glutamate and GABA content in the samples was measured by HPLC coupled with fluorometric  
24 detection (FP-2020 Plus fluorimeter, Jasco, Tokyo, Japan) after precolumn derivatization with o-  
25 phthaldialdehyde/mercaptoethanol (OPA) reagent(70). Thirty microliters of OPA reagent were  
26 automatically added to 28 µL sample by a refrigerated autosampler kept at 4C° (Triathlon, Spark  
27 Holland, Emmen, The Netherlands). Fifty microliters of the mixture were injected onto a 5-C18  
28 Hypersil ODS column (3 X 100 mm; Thermo-Fisher, USA) perfused at 0.48 mL/min (Jasco PU-  
29 2089 Plus Quaternary Pump; Jasco, Tokyo, Japan) with a mobile phase containing 0.1 M sodium  
30 acetate, 10% methanol, 2.2% tetrahydrofuran (pH 6.5). Chromatograms were acquired and analysed  
31 using a ChromNav software (Jasco, Tokyo, Japan). Under these conditions, the limits of detection  
32 for glutamate and GABA were ~1 nM and ~0.5 nM, and their retention times ~3.5 min and ~18.0  
33 min, respectively.

### 35 **Multiple-Layer Perceptrons**

36 Each Multiple-layer Perceptron (MLP) had the same architecture rule: 3 neurons as input, 3 neurons  
37 in the hidden layer and 3 neurons as output. Activation function of neurons was the hyperbolic  
38 tangent. Each network was trained over 1,000 presentations of a subset of the dataset. We used as  
39 error measure the mean square of differences between the expected output and the actual output.  
40 Our implementation comprises two parameters: a learning rate set at 0.05 (regulating the learning  
41 speed), and a momentum set at 0.05 (introducing purposefully a conservatism bias). Prior to  
42 learning, inputs were first scaled and centered (z scoring) in order to avoid dimensionality issues  
43 and then normalized between -0.5 and 0.5. For every combination of 3 variables used as inputs, 50  
44 instances of MLP were trained with different subsets of the dataset. 80% of available data has  
45 been used for learning and the remaining 20% for testing the performance of the network (elements  
46 of each subset were randomly (and uniformly) drawn for each network). The performance from a  
47 given set of input variables was the mean of the error of the 50 instances of MLP that had data for  
48 these variables as inputs. Code was written using Python and the Python scientific stack(71-73)  
49 (Jones, 2001; Walt, 2011; Hunter, 2007). The code is fully available here (DOI:  
50 10.5281/zenodo.1240558). Computation has been done using the Avakas cluster of the Mesocentre  
51 de Calcul Intensif Aquitaine (MCIA). Rank-rank hypergeometric overlap (RRHO) test was  
52 performed as previously described(74) using RRHO package (1.14.0) in R(75) on variable list after  
53 ranking between experimental groups. Plotting was made using matplotlib in Python environment.  
54 The association metric was based on lift calculation. Let a and b be the two variables and  $n_x$  the  
55 number of combinations including variable x and n the total number of combinations considered in  
56 the analysis. Lift calculation was then:

$$Lift_{ab} = \frac{n_{ab}/n_b}{n_a/n}$$

57  
58 The lift calculation was then corrected for performance to avoid selection of detrimental association  
59 by being divided by the mean prediction error of the duo.

## 60 61 **Quantification and statistical analysis**

62 Regarding the data analysis for FTIR microspectroscopy, spectra were analyzed by Principal  
63 Component Analysis (PCA). PCA is a multivariate statistical analysis technique that captures  
64 independent sources of variance in the data and represents them in Principal Components  
65 (eigenvectors) that carry the underlying spectral information and in a Score plot that shows the  
66 relation between spectra and can be used to cluster the data based on the spectral information. PCA  
67 were performed in The UnscramblerX 10.3 (Camo Software) using the SVD algorithm with  
68 leverage correction. Two series of preprocessing were applied prior to PCA and compared. Spectra

69 were either baseline corrected in the amide I region between 1590 and 1700  $\text{cm}^{-1}$  and vector  
70 normalized, or their second derivatives were computed and vector normalized.

71 Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego,  
72 CA). For all experiments, comparisons among means were performed by using One-way analysis  
73 of variance (ANOVA) followed, if appropriate, by a pairwise comparison between means by Tukey  
74 *post-hoc* analysis. All values are expressed as the mean $\pm$ standard error of the mean. Size effect was  
75 assessed with Cohen's d analysis. In all analyses, statistical significance was set at  $p < 0.05$ .

76

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72 performed behavioral analysis. M.G. set up the actimetry behavioral platform. S.D., A.P. and P.A.  
73 performed histologic and immunohistochemical analysis of the data. S.D., A.P. and M.L.A.  
74 performed imaging experiments. E.D. performed electron microscopy analysis. F.L., M.L.A. and  
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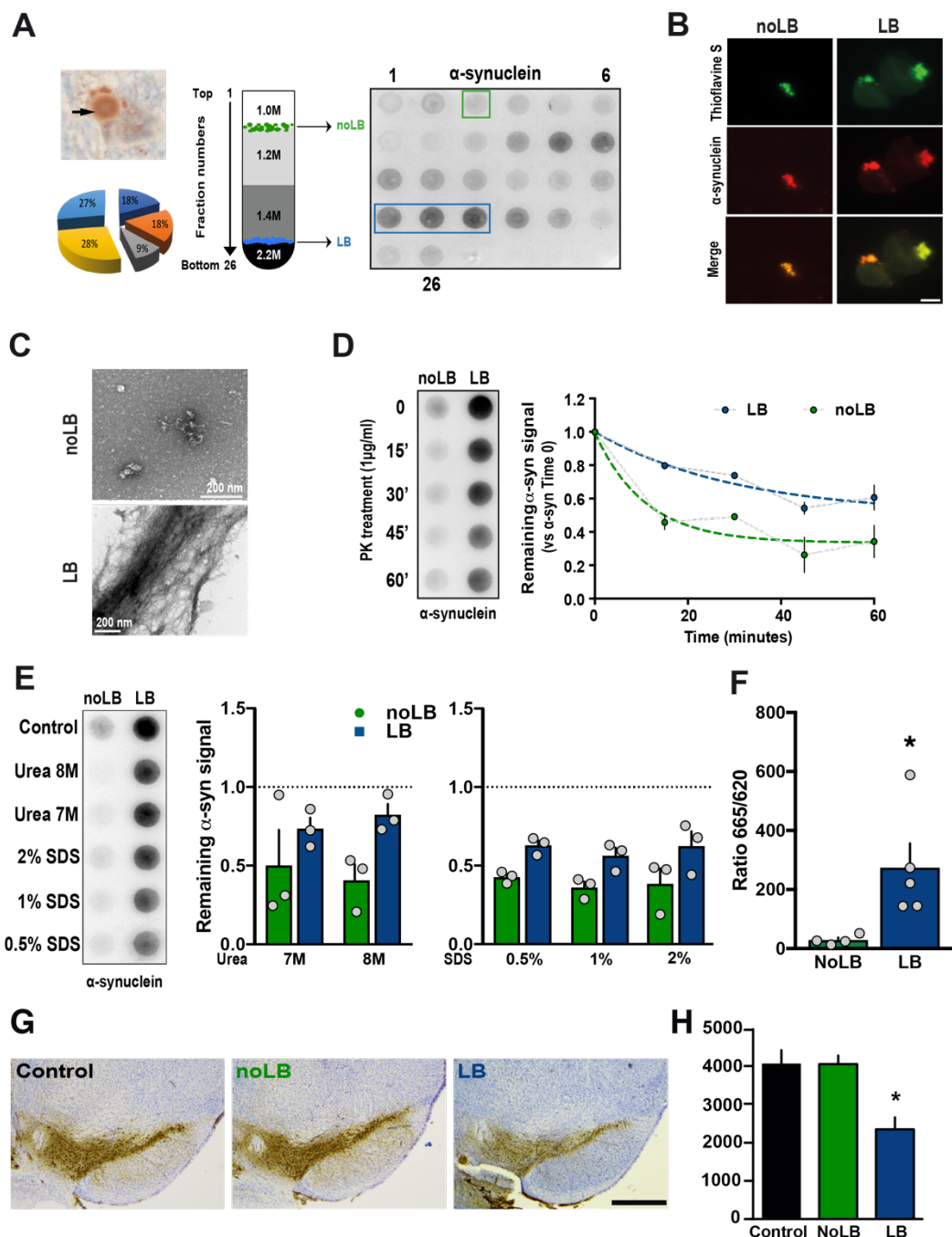
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88 and because the entire data set is fully disclosed. Further information and requests for examples  
89 should be directed to and will be fulfilled by the Corresponding Contacts. Hyperlink to the machine-  
90 learning code (10.5281/zenodo.1240558) is provided  
91 (<https://zenodo.org/record/1240558#.XC8pqy17Su4>).

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94 FIGURES

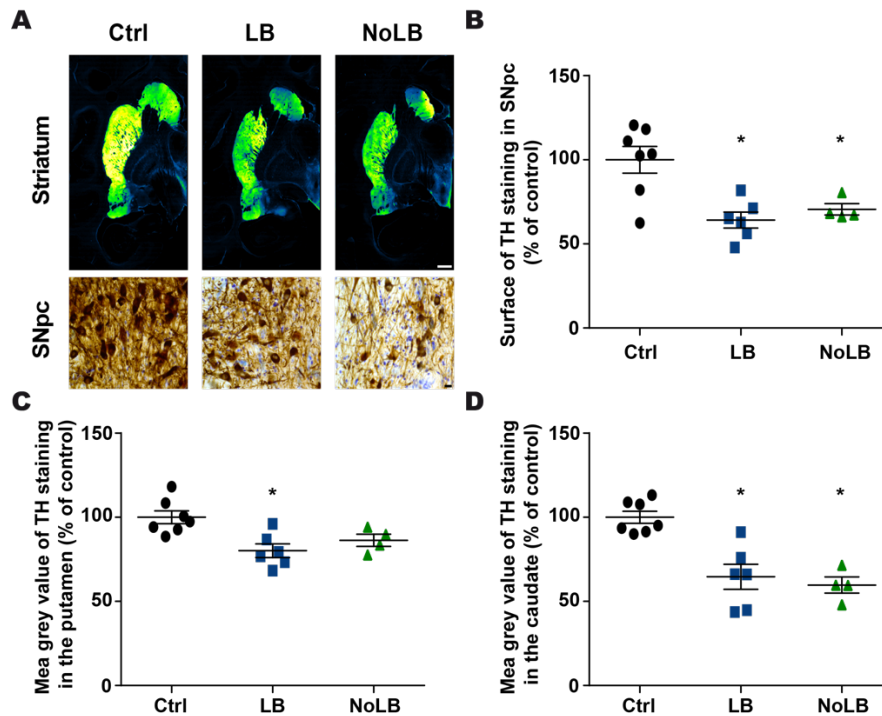


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97 **Fig. 1. Purification and characterization of Lewy bodies (LB) and noLB inocula from**  
 98 **Parkinson disease (PD) brains. (A, left)** Immunohistochemistry image of  $\alpha$ -synuclein–positive  
 99 LB (arrows) in nigral postmortem brain samples (PD #1;  $\alpha$ -synuclein in brown, neuromelanin in  
 00 dark-brown) before sucrose gradient purification. The pie chart indicates the relative contribution  
 01 of the 5 patients to the final pool of LB and noLB inocula (**A, middle**) Schematic representation of  
 02 the sucrose gradient fractionation procedure used to purify LB/noLB-containing fractions from

03 freshly frozen postmortem nigral brain tissue of 5 sporadic PD patients. **(A, right)** Filter retardation  
04 assay probed with a human  $\alpha$ -synuclein antibody to assess the presence of  $\alpha$ -synuclein aggregates  
05 in the different fractions obtained by sucrose gradient fractionation from freshly frozen postmortem  
06 nigral brain tissue from sporadic PD patients (PD #1). Green rectangle indicates noLB-containing  
07 fraction and blue rectangle highlights LB-containing fraction selected to prepare the mixture used  
08 for injections. **(B)** Confocal examination of purified noLB and LB fractions with  $\alpha$ -syn  
09 immunofluorescence (red) and thioflavin S staining (green). Both LB and noLB present thioflavin  
10 S-positive aggregates but much smaller in noLB fractions. Scale bar = 10 $\mu$ m. **(C)** Ultrastructural  
11 examination of noLB and LB fractions by electron microscopy showing massive fibrils in LB  
12 fractions while noLB fractions contain, besides soluble  $\alpha$ -syn, some punctiform small size  
13 aggregates. **(D)** NoLB and LB fractions derived from PD brains (left panel) were treated with 1  
14  $\mu$ g/ml proteinase K for 0, 15, 30, 45 and 60 min and analyzed by immunoblotting with syn211  
15 antibody. The EC50 value was determined as the concentration at which this ratio is decreased by  
16 50%. The corresponding EC50 value for LB (>60 min) was approximately fourfold greater than  
17 with noLB (15.23 min) **(E)** NoLB and LB fractions were treated for 6h with  
18 increasing concentrations of either urea or SDS or buffer as control. Syn211 was used to detect the  
19 forms of  $\alpha$ -synuclein. The LB fractions appear to be more resistant to breakdown compared with  
20 noLB fractions in both urea ( $F_{(1,8)}=6.063$ ,  $p=0.0392$ ) and SDS treatments ( $F_{(1,12)}=17.41$ ,  $p=0.0013$ ).  
21 The dotted line show levels of control fractions. Comparison were made using Two-Way ANOVA.  
22 **(F)** TR-FRET immunoassay analysis of noLB and LB fractions. Fluorescence measurements were  
23 taken 20h after antibody. Analysis by unpaired Student's t-test ( $t_{(7)}=2,623$ ,  $p=0,0343$ ). \*:  $P<0.05$ .  
24 Mean  $\pm$  SEM,  $n=4-5$ . **(G)** Representative pictures of tyrosine hydroxylase (TH)-positive substantia  
25 nigra pars compacta (SNpc) neurons (brown; Nissl staining in purple) in non-injected, noLB or LB-  
26 injected mice at 4 months after injections. Scale bars=500 $\mu$ m. **(H)** Quantification of TH-positive  
27 Substantia Nigra pars compacta (SNpc) neurons by stereology in control, LB- and noLB-injected  
28 mice. Control mice,  $n=10$ , LB-injected mice at 4 months,  $n=10$ , No-LB-injected mice at 4 months,  
29  $n=10$ . One-way ANOVA followed by Tukey test for multiple comparisons. \*:  $p<0.05$  compared  
30 with control and noLB-injected side at 4 months.

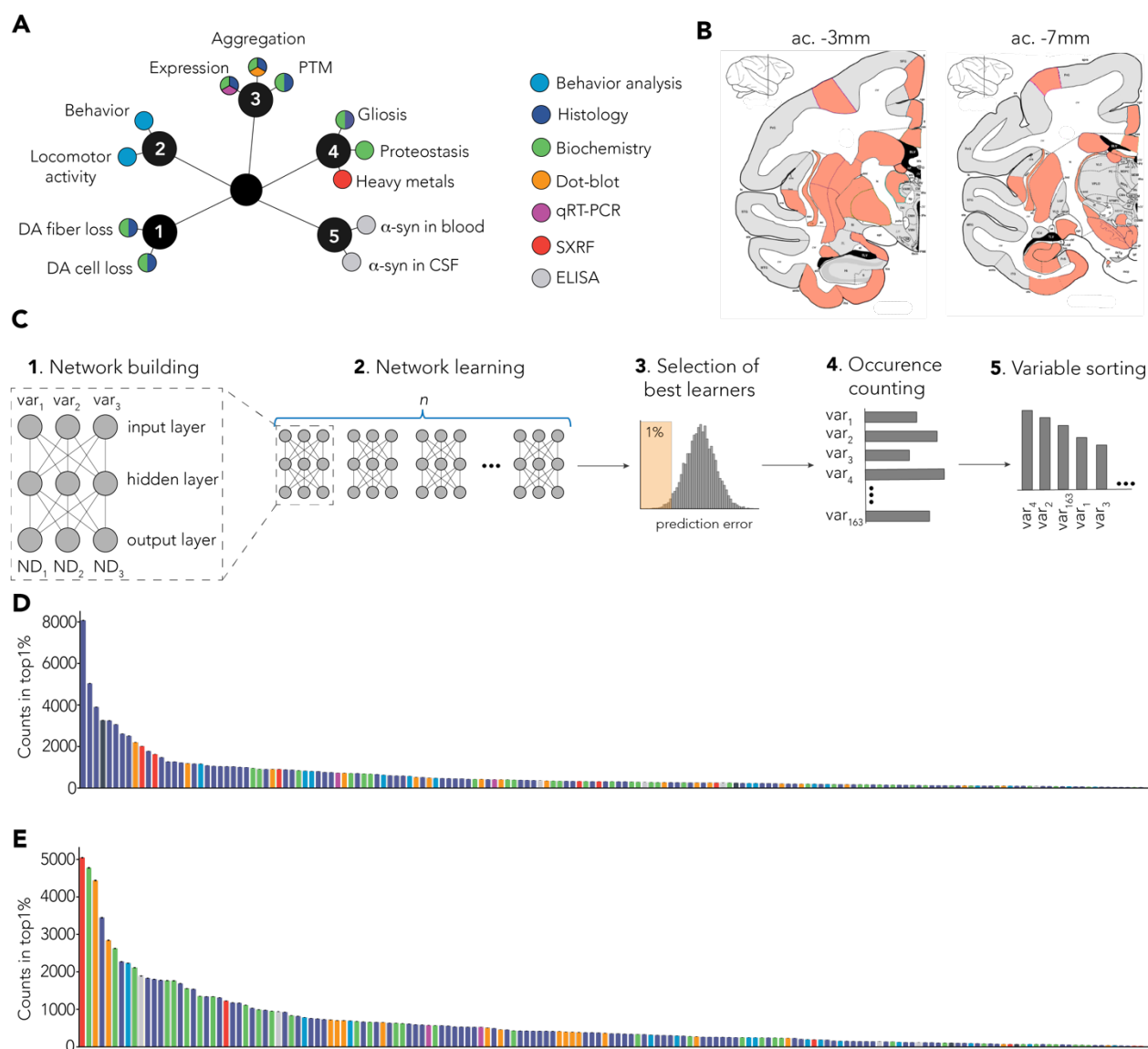


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34 **Fig. 2. Intra-striatal injection of Lewy bodies (LB) and noLB fractions from Parkinson's**  
35 **disease patients induces nigrostriatal neurodegeneration in baboon monkeys.** (A) Tyrosine  
36 hydroxylase (TH) staining at striatum and Substantia Nigra pars compacta (SNpc) levels. A green  
37 fire blue LUT (lookup table) was used to enhance contrast and highlight the difference between  
38 non-injected, LB-injected and noLB-injected baboon monkeys at striatum level. Scale bars = 5mm  
39 (striatum) and 10 $\mu$ m (SNpc). (B) Scatter plot of TH immunostaining in SNpc.  $F_{(2,14)}=9.439$ ,  
40  $p=0.0025$ . Control vs LB-injected:  $p=0.0029$ . Control vs noLB- injected:  $p=0.0248$ . (C, D) Scatter  
41 plots of mean grey values of striatal TH immunoreactivity in the putamen ( $F_{(2,14)}=7.313$ ,  $p=0.0067$ ;  
42 Control vs LB-injected:  $p=0.0059$ ) (C) and in the caudate ( $F_{(2,14)}=16.25$ ,  $p=0.0002$ ; Control vs LB-  
43 injected:  $p=0.0008$ ; Control vs noLB- injected:  $p=0.0008$ ) (D) in non- injected, LB-injected and  
44 noLB-injected baboon monkeys. The horizontal line indicates the average value per group  $\pm$  SEM  
45 ( $n=7$  from control animals;  $n=6$  for LB-injected animals;  $n=4$  for noLB-injected animals).  
46 Comparison were made using One-Way ANOVA and Tukey's correction for multiple comparison.  
47 \* $p < 0.05$  compared with control animals.

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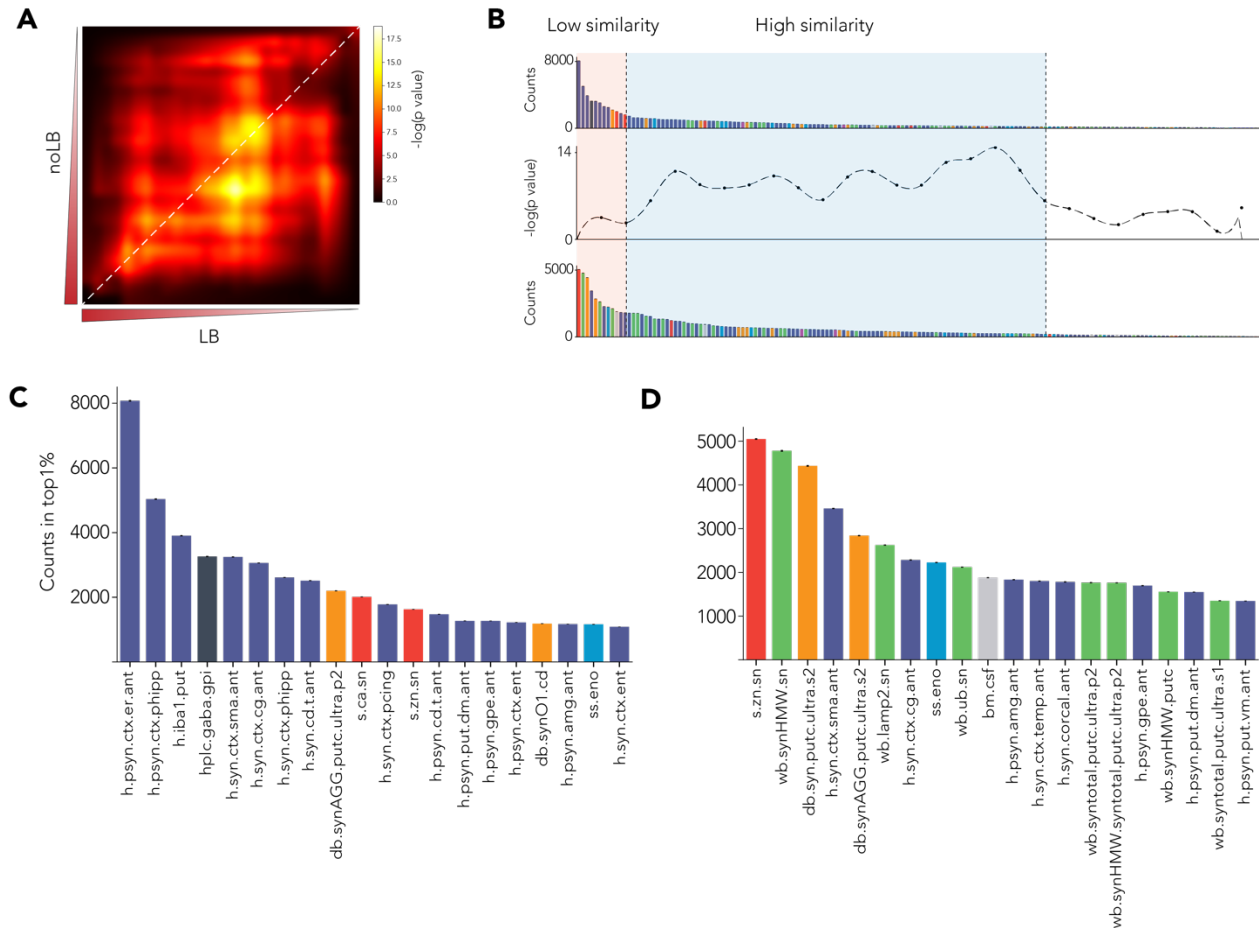


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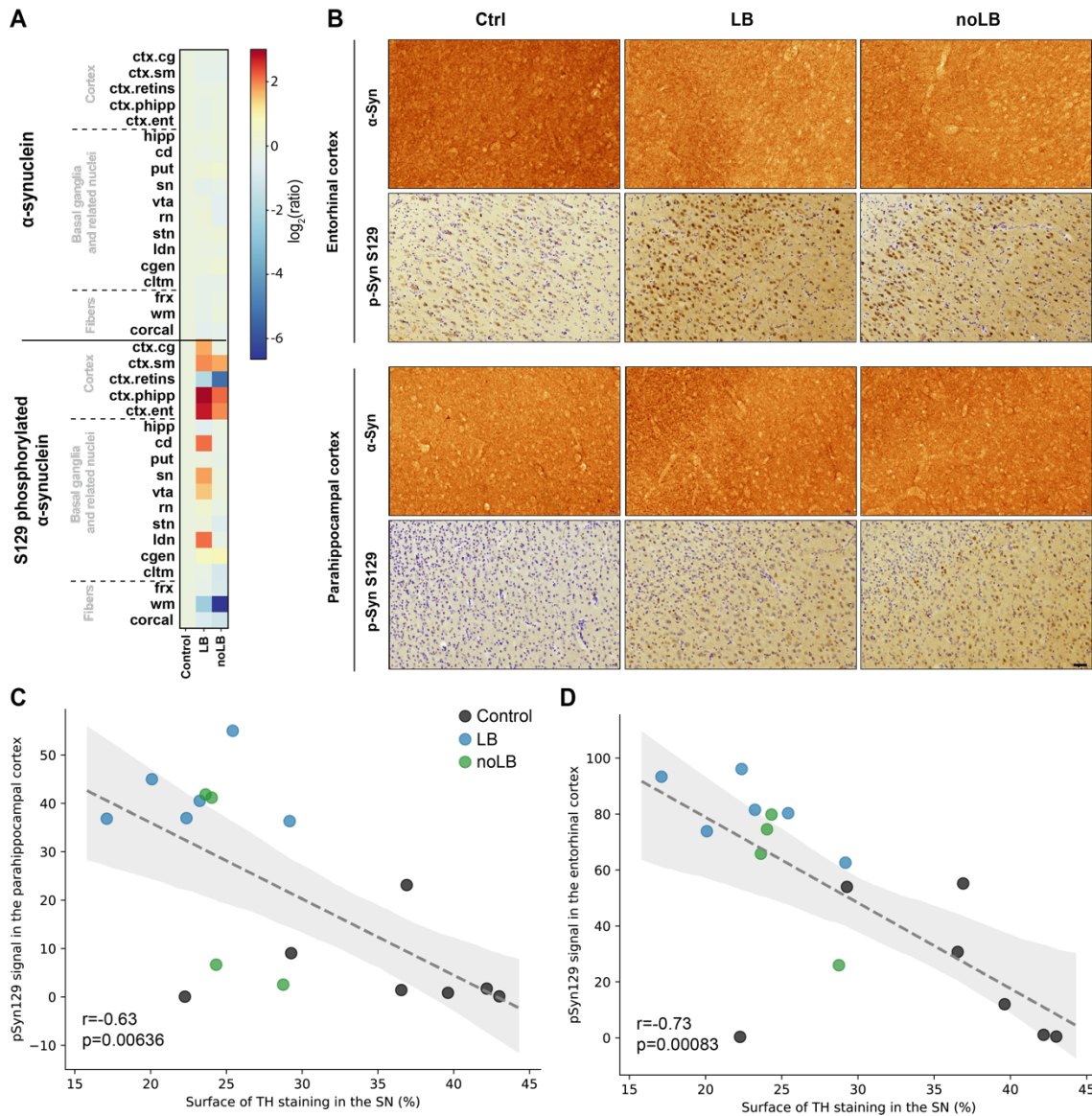
51 **Fig. 3. Multiple-layer perceptron (MLP)-based identification of specific signature.** (A) Several  
 52 endpoints (n=180) were measured using multiple methods (colors). Endpoints can be grouped as  
 53 clusters: 1. Dopaminergic degeneration, 2. Behavior, 3.  $\alpha$ -syn-related pathology. 4. Non- $\alpha$ -syn  
 54 related pathology. 5. Putative biomarkers. (B) Multiple brain regions (n=40) were investigated from  
 55 coronal sections at 2 levels: anterior commissure (ac.) -3mm (striatum, entorhinal cortex) and -7mm  
 56 (SNpc, hippocampus). (C) Detailed methodology. 1. Representative scheme of one MLP predicting  
 57 3 neurodegeneration-related variables (ND<sub>1</sub>, ND<sub>2</sub>, ND<sub>3</sub>) with 3 experimental variables as input  
 58 (var<sub>1</sub>, var<sub>2</sub>, var<sub>3</sub>). Out of the 180 variables measured in total, 163 were used as inputs for the MLP.  
 59 2. One MLP was trained for every unique combination of 3 variables. 3. Combinations were ranked  
 60 based on their prediction error and top1% were selected for further analysis. 4. Combinations were  
 61 deconvoluted to extract single variables and count occurrence of individual variables. 5. Variables

62 were sorted based on the number of occurrences in the top1% of the best combination. **(D)** Raw  
 63 ranking obtained for LB-injected animals. Color code highlights measurement methods as in A. **(E)**  
 64 Raw ranking obtained for noLB-injected animals. Color code highlights measurement methods as  
 65 in A.



68 **Fig. 4. Direct comparison of MLP-derived signatures shows specific pattern between**  
 69 **experiment groups. (A)** Rank-rank hypergeometric overlap (RRHO) test between variable sorting  
 70 **of LB and noLB-injected animals. Highly enriched variables are in the lower left corner. Diagonal**  
 71 **(highlighted by a red dashed line) was extracted to do a bin-to-bin comparison between LB and**  
 72 **noLB signatures. (B)** Signatures were aligned with RRHO and show low similarity in highly  
 73 **enriched variables (light orange background) and higher similarity for lower rank variables (pale**  
 74 **blue background). (C, D)** First 20 enriched variables for both LB-injected animals **(C)** and noLB-  
 75 **injected animals **(D)**. Color code is similar to Fig. 2A. Detailed of variable names can be found in**  
 76 **Table S1. Bars are mean +/- 99% confidence interval estimated by bootstrap.**

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82 **Fig. 5. Levels of  $\alpha$ -synuclein and phosphorylated  $\alpha$ -synuclein in different brain regions. (A)**

83 Heat map representing the surface of  $\alpha$ -synuclein ( $\alpha$ -syn) and S129 phosphorylated  $\alpha$ -syn

84 immunostaining intensity in the brain of non-inoculated, LB-inoculated and noLB-inoculated

85 baboon monkeys. The heat maps show all brain regions measured and are organized according in

86 3 main groups: cortical, basal ganglia and sub-cortical areas. From top to bottom: cingulate cortex

87 (*ctx.cg*), sensorimotor cortex (*ctx.sm*), retro-insular cortex (*ctx.retins*), parahippocampal cortex

88 (*ctx.phipp*), entorhinal cortex (*ctx.ent*), hippocampus (*hipp*), caudate nucleus (*cd*), putamen (*put*),

89 substantia nigra (*sn*), ventral tegmental area (*vta*), red nucleus (*rn*), subthalamic nucleus (*stn*),

90 lateral dorsal nucleus (*ldn*), lateral geniculate nucleus (*cgen*), claustrum (*cltm*), fornix (*frx*), white

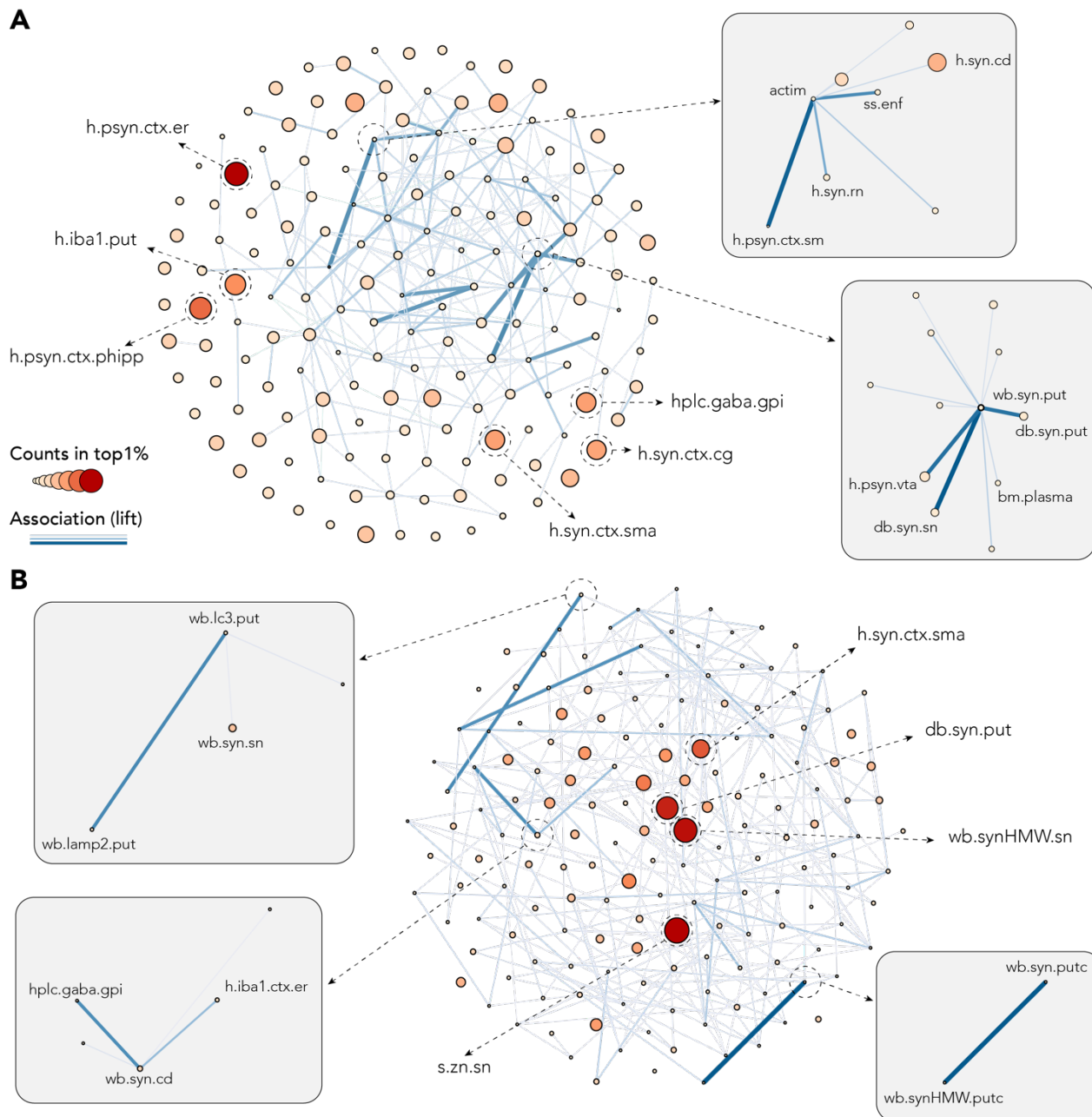
91 matter (*wm*), corpus callosum (*corcal*). The color bars represent the  $\log_2$  value of the ratio of each

92 brain regions. (B) Representative pictures of  $\alpha$ -syn ( $\alpha$ -syn) and phosphorylated  $\alpha$ -syn (pSyn S129)



93 staining in the entorhinal and parahippocampal cortices. (C, D) Correlation between levels of  
94 phosphorylated  $\alpha$ -syn in the parahippocampal cortex (C) and the entorhinal cortex (D) with levels  
95 of TH staining in the substantia nigra. Dotted line indicates the linear regression. Gray area indicates  
96 the 95% confidence interval around of the linear regression.

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01 **Fig. 6. Association metric shows independence of strong predictors and beneficial association**  
02 **of weaker predictors.** Both network plots were build using number of counts in the top1% as node  
03 size and color, and lift (association measure) as edges. To allow better visualization, only 10% of

04 the strongest edges are shown. (A) Network plot for LB-injected animals showing independence  
05 of strong predictors: S129 phosphorylated  $\alpha$ -syn (psyn) in the entorhinal (*h.psyn.ctx.er*) and the  
06 para-hippocampal cortex (*h.psyn.ctx.phipp*), microglia-activation in the putamen (*h.ibal.put*),  $\alpha$ -  
07 syn in the cingulate cortex (*h.syn.ctx.cg*) and the supplementary motor area (*h.syn.ctx.sma*) and  
08 GABA levels in the internal part of the globus pallidus (*hlpc.gaba.gpi*). Upper right box highlights  
09 association between actimetry measure (*actim*) and a scan-sampling measure of body direction  
10 toward a closed environment (*ss.enf*) with  $\alpha$ -syn levels in the caudate nucleus (*h.syn.cd*), the red  
11 nucleus (*h.syn.rn*) and psyn in the sensorimotor cortex (*h.psyn.ctx.sm*). Lower right box highlights  
12 association between pathological  $\alpha$ -syn in the putamen (*wb.syn.put* and *db.syn.put*) and the SNpc  
13 (*db.syn.sn*) as well as psyn in the ventral tegmental area (*h.psyn.vta*) and peripheral levels of  $\alpha$ -syn  
14 in the plasma (*bm.plasma*). (B) Network plot for noLB-injected animals showing independence of  
15 strong predictors: levels of Zn in the SNpc (*s.zn.sn*), pathological  $\alpha$ -syn in the putamen (*db.syn.put*),  
16  $\alpha$ -syn in the supplementary motor area (*h.syn.ctx.sma*) and aggregated  $\alpha$ -syn in the SNpc  
17 (*wb.synHMW.sn*). Upper left box highlights association between autophagosomes (*wb.lc3.put*) and  
18 lysosomes (*wb.lamp2.put*) levels in the putamen and  $\alpha$ -syn in the SNpc (*wb.syn.sn*). Lower left box  
19 highlights association between GABA levels in the internal part of the globus pallidus  
20 (*hlpc.gaba.gpi*),  $\alpha$ -syn in the caudate nucleus (*wb.syn.cd*) and microglia activation in the entorhinal  
21 cortex (*h.ibal.ctx.er*). Lower right box highlights association between soluble (*wb.syn.putc*) and  
22 aggregated (*wb.synHMW.putc*) levels of  $\alpha$ -syn in the putamen.

23

#### 24 **Supplementary Materials:**

25 **Table S1.** List of variables used in multiple-layer perceptron analyses.

26 **Table S2.** Raw data that served for the multiple-layer perceptron analyses for all behavioral,  
27 histological, biochemical, transcriptional and biophysical approaches (applied to several brain  
28 areas, totalizing the quantification of 180 variables for each individual).