Seeded propagation of alpha-synuclein aggregation in mouse brain using protein misfolding cyclic amplification

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21 **Abstract** Alpha-synuclein (α -syn) protein aggregation is associated with several
 22 neurodegenerative disorders collectively referred to as synucleinopathies, including
 23 Parkinson's disease. We used protein misfolding cyclic amplification (PMCA) to study α -syn
 24 aggregation in brain homogenates of wild-type or transgenic mice expressing normal (D line)
 25 or A53T mutant (M83 line) human α -syn. We found that sonication-incubation cycles of M83
 26 mouse brain gradually produce large quantities of SDS-resistant α -syn aggregates, involving
 27 both human and mouse proteins. These PMCA products, containing partially proteinase K
 28 resistant α -syn species, are competent to accelerate the onset of neurological symptoms after
 29 intracerebral inoculation to young M83 mice and to seed aggregate formation of α -syn
 30 following PMCA, including in D and wild-type mouse brain substrates. Our data indicate that
 31 similar to prions, PMCA can reproduce some characteristics of α -syn aggregation and seeded-
 32 propagation *in vitro* in a complex milieu. This opens new opportunities for the molecular
 33 study of synucleinopathies.

34

35 **Key words:** alpha-synuclein, protein misfolding cyclic amplification, aggregation, prion,
 36 seeding

37 **Introduction**

38 Alpha-synuclein (α -syn) is a neuronal protein critically involved in a group of human
 39 neurodegenerative diseases, called synucleinopathies, including Parkinson's disease (PD),
 40 dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). These diseases are
 41 characterized by α -syn intracellular inclusions, in neurons in PD and DLB, presenting as
 42 Lewy bodies and Lewy neurites, or in oligodendrocytes in MSA, presenting as glial
 43 cytoplasmic inclusions. Most cases of these diseases are considered as sporadic, but some
 44 cases can be genetically triggered by duplication or triplication of the α -syn encoding SNCA
 45 gene, leading to constitutive overexpression of the protein, or by mutations in the N-terminal
 46 region of the protein, which favor its oligomerization and aggregation (A30P, E46K or A53T
 47 for instance)(Lazaro et al., 2014). Evidence of somatic SNCA gains in brain, more commonly
 48 in nigral dopaminergic neurons of Parkinson's disease than in controls, and possibly
 49 commonest in some multiple system atrophy cases, was also recently described, suggesting
 50 that this may be a risk factor for sporadic synucleinopathies, or alternatively a result of the
 51 disease process (Mokretar et al., 2018).

52 The detailed molecular pathways leading to α -syn aggregation still remain poorly
 53 understood. However, a primary event leading to α -syn oligomers would involve α -syn self-
 54 interactions in a rate-limiting step of α -syn dimers formation (Krishnan et al., 2003; Roostae
 55 et al., 2013). The initiation and amplification steps of α -syn aggregation can be dramatically
 56 changed by mutations associated with familial forms of PD (Flagmeier et al., 2016), and the
 57 dimer formation was notably shown to be accelerated by the A30P and A53T mutations
 58 (Krishnan et al., 2003).

59 The pathogenesis of synucleinopathies is characterized by a progressive spreading of α -
 60 syn lesions in the patient body, and a number of recent studies suggested that it could involve

61 molecular mechanisms similar to those occurring in prion diseases (for a recent review Steiner
62 et al., , 2018)). In this paradigm, pathological α -syn (α -syn^P) species act as seeds that self-
63 propagate at the expense of the normal protein and spread through interconnected brain
64 regions. Previously, we and others have used a transgenic mouse model overexpressing the
65 human mutated A53T α -syn on a non-knockout mouse α -syn background (called M83)
66 (Giasson et al., 2002) to show that a synucleinopathy can be experimentally accelerated *in*
67 *vivo* following inoculation of both M83^{+/+} and M83^{+/-} young mice with pathological α -syn
68 assemblies of different origin (from old M83 mice, humans with MSA or recombinant fibrils)
69 and inoculated via different routes, including intracerebral and peripheral (intramuscular,
70 intra-peritoneal, intrasciatic) routes (Ayers et al., 2017; Ayers et al., 2018; Breid et al., 2016;
71 Luk et al., 2012; Mougenot et al., 2012; Prusiner et al., 2015; Rutherford et al., 2017; Sacino
72 et al., 2014a; Sacino et al., 2014b; Sargent et al., 2017; Watts et al., 2013; Woerman et al.,
73 2018).

74 We recently showed that the spreading of pathological α -syn in the M83 mouse brain
75 can be monitored using an ELISA test, which specifically recognizes α -syn^P in some brain
76 regions (mainly brain stem and midbrain), and in the spinal cords of sick M83 mice, which
77 are characterized by neuronal α -syn inclusions (Betemps et al., 2014; Giasson et al., 2002;
78 Sargent et al., 2017). The precise molecular species recognized by this ELISA test remain to
79 be determined, but immunoreactivity is observed with antibodies against epitopes in the C- or,
80 to a lesser extent, N-terminal ends of the protein that are known to represent less structured
81 regions of the protein compared to the central region, which is involved in the formation of β -
82 sheet structural motifs in amyloid fibrils (Riek & Eisenberg, 2016).

83 Protein misfolding cyclic amplification (PMCA) provides faithful replication of
84 mammalian prions *in vitro*. Presumably because it operates in a crude brain milieu containing

85 potential cellular co-factors necessary for prions to multiply, PMCA has the unique ability to
 86 generate *bona fide* infectious prions (Castilla et al., 2005). The method, conceptually based on
 87 the nucleation-dependent polymerization model of prion replication, allows conversion of the
 88 cellular prion protein from a normal brain substrate into an aggregated form, using repeated
 89 cycles of sonication and incubation, after seeding with a small quantity of a sample containing
 90 the pathological prion protein (Saborio et al., 2001). In the last few years, our laboratory has
 91 developed a highly efficient PMCA methodology, which allowed for the first time the
 92 preclinical detection of prions in the plasma of patients incubating variant Creutzfeldt-Jakob
 93 disease (Bougard et al., 2016).

94 Regarding the α -syn protein, the use of PMCA has been reported on α -syn of
 95 recombinant origin (Fairfoul et al., 2016; Herva et al., 2014; Jung et al., 2017; Roostae et al.,
 96 2013; Shah Nawaz et al., 2017) but not to our knowledge on brain α -syn (Paciotti et al., 2018).
 97 In this study we developed α -syn PMCA in mouse brain homogenates, including M83
 98 (Giasson et al., 2002) and D (Masliah et al., 2000) transgenic mice expressing A53T-mutant
 99 and wild-type human α -syn respectively, as well as C57Bl/6 wild-type mice. We demonstrate
 100 for the first time the *in vitro* generation and seeded-propagation of α -syn aggregates in mouse
 101 brain tissue, with subsequent transmission of an experimental synucleinopathy in M83 mice
 102 by intracerebral inoculation.

103

104 **Results**

105 To determine the effects of PMCA on the α -syn protein in mouse brain, we prepared brain
 106 homogenates (10% wt/vol) in conversion buffer from different mouse lines, including: (i)
 107 M83 mice expressing A53T-mutated human α -syn (homozygous (M83^{+/+}) or hemizygous
 108 (M83^{+/-}) for the transgene), (ii) D (^{+/+}) mice expressing human wild-type α -syn, (iii) C57Bl/6

wild-type mice (C57Bl/6-WT) and (iv) C57Bl/6 mice presenting a deletion of the α -syn gene (C57Bl/6-KO) (Specht & Schoepfer, 2001). These brain homogenates were then submitted to a 72h (144 cycles) PMCA procedure using a microplate sonicator (**Figure 1A**). In the meantime, the same brain homogenates were either only incubated at 37°C (“standing” condition) or under constant shaking at 1000 rpm (“shaking” condition). Unexpectedly, after 144 cycles of sonication-incubation, we observed the abundant formation of, at least partially, SDS-resistant α -syn aggregates, specifically in brain homogenates from M83 mice, as shown by Western blot detection using the C-20R antibody (**Figure 1A**). This showed bands consistent with α -syn dimers and trimers, as well as higher molecular weight species, some of which were retained in the stacking of the gels. By contrast, a single band corresponding to monomeric α -syn was detected in D or C57Bl/6-WT mouse brains following the same PMCA procedure. The “*de novo*” aggregation of α -syn from M83 brain required the sonication process, as only monomeric α -syn was detected in the standing and shaking conditions and in the initial “frozen” brain samples (**Figure 1A**). The sonication-induced formation of α -syn aggregates was enhanced by increased α -syn levels, as shown by comparisons of brain homogenates from M83^{+/+} and M83^{+/-} mice, as well as of 10% *versus* 5% wt/vol concentrations (**Figure 1B**). This also showed an increase of α -syn aggregation with increasing numbers of cycles of sonication-incubation and this required the addition of Teflon beads into the test tube (data not shown). We did not observe α -syn aggregation from M83^{+/-} 5% wt/vol homogenates after up to 96 cycles of sonication-incubation, which enabled us to apply these experimental conditions in the following experiments when using the M83^{+/-} mouse brain as a substrate in seeding experiments. Using an antibody specifically recognizing β -synuclein did not reveal any aggregation of this protein in the four mouse brain substrates following PMCA (**Figure 1C**). To further characterize α -syn species in the PMCA products, we performed limited *in vitro* proteolysis of the samples using proteinase K (PK) digestion

(**Figure 2**). After PMCA, α -syn from M83^{+/-} brain resists PK treatment at up to 5 μ g/ml for 30 min, whereas without PMCA it is fully degraded even at a 0.5 μ g/ml PK concentration. In D and C57Bl/6-WT brain homogenates, either before or after PMCA, α -syn is similarly fully degraded at 0.5 μ g/ml PK concentration. Thus, repeated cycles of sonication-incubation specifically induce the formation of partially PK-resistant α -syn aggregates in M83 mouse brain.

We next investigated whether the *de novo* PMCA-generated α -syn aggregates from M83 mice would act as seeds for the aggregation of α -syn when introduced in PMCA reactions (**Figure 3**). To test this hypothesis, we serially diluted 10-fold (from 10^{-3} to 10^{-6}) a 144 cycle PMCA product from M83^{+/-} mice harboring abundant α -syn aggregates. These dilutions were then used to seed M83^{+/-}, D, C57Bl/6-WT and C57Bl/6-KO brain substrates and the resulting mixtures were subjected to PMCA. As shown in the C57Bl/6-KO substrate, α -syn aggregates attributable to the seed were detected in the 10^{-3} and to a lesser extent in the 10^{-4} dilutions after PMCA. However, in all other substrates, abundant α -syn aggregates were still detected at the 10^{-6} dilution, whereas no signal was observed in unseeded PMCA reactions (NBH lanes) or in control PMCA reactions seeded with a 10^{-3} dilution from a M83^{+/+} brain that was not previously subjected to PMCA (CTL lanes) (**Figure 3A,B**). To rule out the possibility that this seeding effect could be attributable to other factors than aggregated α -syn itself, some of which could have been generated during the process of sonication-incubation of brain homogenates to generate the seed, we similarly seeded PMCA reactions with the products of 144 PMCA cycles from D, C57Bl/6-WT or C57Bl/6-KO brain substrates that were previously shown to be free of detectable α -syn aggregates. We were unable to detect any α -syn aggregation in M83^{+/-} substrate following PMCA after seeding with these samples (data not shown). Altogether, these experiments clearly showed that the addition of α -syn aggregates produced by PMCA from M83 brain was able to seed α -syn aggregation, including in D and

C57Bl/6-WT brain substrates, which otherwise do not show detectable *de novo* α -syn aggregation in the absence of seeding. Next, we examined the PMCA samples using an ELISA test, as previously described for the specific identification of the pathological α -syn in sick M83 mice (Betemps et al., 2014; Sargent et al., 2017), using the same C-20R antibody for α -syn detection (**Figure 3C,D**). Overall, we found a good correlation between ELISA and Western blot results consistent with the detection of PMCA-generated α -syn aggregates. These results emphasize the fact that this ELISA test likely identifies at least in part aggregated α -syn species in the mouse brain, offering an additional and convenient method for measuring α -syn aggregates in a PMCA sample.

To examine the respective contributions of human and mouse α -syn proteins in the formation of the α -syn aggregates after seeding with *de novo* M83 PMCA products, we performed Western blot analyses using antibodies specific to either human (MJFR1) or mouse (D37A6) α -syn (**Figure 4A-C**). We found a major difference in the composition of α -syn aggregates in D and M83^{+/-} substrates. In D substrate, detection of human α -syn with the MJFR1 antibody showed a strong labeling for the α -syn monomer, whereas α -syn dimers and trimers were labeled as in C57Bl/6-KO and C57Bl/6-WT substrate, which correspond to the human α -syn seed input from M83 mice. Using the D37A6 antibody against mouse α -syn showed similar results in C57Bl/6-WT and D substrates, whereas no signal was detected in C57Bl/6-KO brain substrate. The reverse situation was observed in the M83^{+/-} substrate, in which both human α -syn aggregates and monomers were strongly labeled by the MJFR1 antibody, whereas, unexpectedly, mouse α -syn detection with D37A6 was very low in comparison to C57Bl/6-WT and D substrates, including for the mouse α -syn monomer. Because similar amounts of mouse α -syn monomer were detected with D37A6 in M83^{+/-}, D and C57Bl/6-WT brain substrates before PMCA (data not shown), this points to a strong

effect of PMCA on this decreased detection of mouse α -syn in these Western blot conditions, specifically in the M83 brain substrate. These samples were also examined by ELISA with α -syn detection using MJFR1, D37A6 or C-20R antibodies (**Figure 4D**). The results showed that the human protein is recognized much better from the M83 than from the D brain substrate, although increased immunoreactivity is clearly detected in the D substrate (but only after seeding); in contrast, the levels of mouse α -syn species recognized by D37A6 are roughly comparable in the three brain substrates (M83, D and C57Bl/6-WT), including in the M83 samples that show decreased detection in Western blot after the PMCA reaction. Immunoreactivity is extremely low in samples that do not show α -syn aggregates by Western blot (NBH). Altogether, these data suggest a higher aggregation of human α -syn in the M83 brain substrate compared to the D brain substrate and a similar aggregation of mouse α -syn in M83, D or C57Bl/6-WT brain substrates.

Having shown that PMCA can propagate α -syn aggregation after *in vitro* seeding of mouse brain substrates, including that of C57Bl/6-WT mice, we examined the seeding capacities of M83 mouse brain following development of the characteristic M83 clinical disease. Serial 10-fold dilutions of a 10% whole brain homogenate were prepared from a sick M83^{+/+} mouse at the terminal stage of the disease before seeding in the different substrates (M83^{+/+}, D, C57Bl/6-WT and C57Bl/6-KO) (**Figure 5A**). Following PMCA, SDS-resistant α -syn dimers and trimers were detected by Western blot in all substrates except for the C57Bl/6-KO control, which shows that these α -syn species are derived from the substrate and not from the seed. However, compared to previous experiments using a *de novo* PMCA-generated seed from M83^{+/+} substrate, the detection sensitivity achieved with this endogenous seed from sick M83 brain was lower. To increase the detection limit, we performed an additional PMCA round in the non-transgenic C57Bl/6-WT substrate, a procedure generally used in PMCA

studies for prions that can achieve a very high level of sensitivity. Aliquots of each dilution from the first PMCA round were mixed with fresh substrate and submitted to a new PMCA round. As shown in **Figure 5B**, we could detect a seeding activity up to the 10^{-4} and 10^{-6} dilutions at the first and second PMCA round respectively from a sick M83^{+/+} mouse brain homogenate. Importantly, no α -syn aggregates were detected after two PMCA rounds of 144 cycles in either the unseeded C57Bl/6-WT brain substrate (**Figure 5B**) or in the C57Bl/6-KO brain substrate seeded by M83 brain tissue (data not shown).

We next tested the capacity of this PMCA assay to distinguish between healthy and sick M83 mice in a panel of samples from healthy (uninoculated ≤ 180 days old) (n=13) or sick (n=9) M83^{+/+} mice. The different origins, clinical and pathological status and ages of these mice are summarized in **Table 1**. These experiments, performed from whole brain homogenates at dilutions 10^{-3} and 10^{-4} and using M83^{+/+} or C57Bl/6-WT brain substrates, readily discriminated sick and healthy M83 mice (**Table 1 and Figure 6A,B**). All the brains of the sick mice were shown to contain high levels of Ser129 phosphorylated α -syn by ELISA (**Figure 6C**). From unchallenged and healthy M83 mice below the age of 6 months, no α -syn aggregates were observed after a single PMCA round on either brain substrate; however, some of these samples (from mice 110-146 days old) showed a seeding activity which was identified by a second PMCA round performed with the C57Bl/6-WT brain substrate (**Table 1 and Figure 6A**). Very low levels of immunoreactivity were detected by ELISA with the antibody against Ser129 phosphorylated α -syn, in some of these mice and at the lower dilution tested (**Figure 6C**).

Because α -syn neuropathology is much more pronounced in the caudal brain regions (brain stem and midbrain) of sick M83 mice, we compared the PMCA seeding activities in different brain regions of two M83 mice at the terminal stage of the disease (573 or 670 days old)(**Figure 7A**). The highest PMCA seeding activities were detected in the brain stem and

midbrain of these mice. Intermediate results were obtained in the cerebral cortex, striatum and cerebellum, whereas the lowest seeding activities were found in the hippocampus and olfactory bulb, although α -syn aggregates could be detected after two PMCA rounds at the 10^{-3} dilution in these regions (data not shown). These PMCA results are in good agreement with the results of direct detection of Ser129 phosphorylated α -syn by ELISA in these same brain regions, as illustrated in **Figure 7B**. Thus, we found a positive correlation between the known degree of α -syn neuropathology and the PMCA seeding activity in different regions of the brain of sick M83 mice.

We finally addressed the question as to whether the PMCA products can seed α -syn pathology *in vivo*. For this, young M83^{+/+} mice were inoculated intracerebrally with samples obtained after a procedure involving 144 PMCA cycles in the M83^{+/+}, M83^{+/-} or C57Bl/6-KO (2 months old) brain substrates. As additional controls, we inoculated the M83^{+/+} mouse brain substrate without sonication (i.e. standing or shaking conditions). Individual survival data during a one-year follow up after stereotaxic intracerebral challenge are shown in **Figure 8**. In M83 mice, the disease was strikingly accelerated by the inoculation of the sonicated M83 (^{+/+} or ^{+/-}) brain samples, with all the mice showing the typical clinical signs of M83 mice (Giasson et al., 2002; Sargent et al., 2017), before the age of 6 months. The disease was confirmed by ELISA analysis showing detection of Ser129 phosphorylated pathological α -syn in the spinal cords of these mice, as previously described (Betemps et al., 2014; Sargent et al., 2017). In contrast, the inocula prepared from M83 brain in shaking or in standing conditions, or from sonicated C57Bl/6-KO brain homogenate, failed to accelerate the disease in M83 mice, which only began to develop the disease after the age of 9 months, as has been described for this model (Giasson et al., 2002; Mougenot et al., 2012). Thus, the M83-derived PMCA samples that contained α -syn aggregates strikingly accelerated the M83 disease, as

previously reported with brain homogenates from sick M83 mice (Luk et al., 2012; Mougenot et al., 2012).

Discussion

During the last decade, studies in cellular and animal experimental models have suggested that prion-like behavior of α -syn is involved in the pathogenesis of synucleinopathies (Ayers et al., 2018; Betemps et al., 2014; Luk et al., 2012; Luk et al., 2009; Mougenot et al., 2012; Prusiner et al., 2015). In the present study, we tested the hypothesis that the sonication-based version of the PMCA technology, developed by Claudio Soto's group in the early 2000s for the sensitive amplification of the prion protein aggregation (Saborio et al., 2001), could be used to study α -syn prion-like properties. This PMCA version operates in a crude brain milieu containing all potential cellular cofactors that may be involved in the α -syn aggregation process *in vivo*. Our study demonstrates, for the first time, the *in vitro* generation and seeded-propagation of α -syn aggregates in a biological complex matrix of brain homogenate. Furthermore, this is the first report showing efficient PMCA in a brain substrate of a protein distinct from the prion protein. This key finding thus adds to the growing list of arguments supporting the idea that α -syn can act in the same way as a prion. Furthermore, our bioassay experiments revealed that the PMCA-generated α -syn aggregates were biologically competent to seed α -syn neuropathology *in vivo*.

Our study first revealed that repetition of PMCA sonication-incubation cycles provokes the spontaneous formation of partially PK-resistant α -syn aggregates in the M83 mouse brain substrate, identified by Western blotting in denaturing conditions. This *de novo* α -syn aggregation was not observed in the other D or C57Bl/6-WT substrates in our experimental conditions, suggesting the involvement of mutant human α -syn expressed by M83 mice.

Consistently, PMCA α -syn aggregation kinetics in the M83 substrate were correlated to the amount of A53T-mutated human protein, as evidenced by the comparisons of M83^{+/+} and M83^{+/-} substrates. We confirmed the crucial effects of sonication in this phenomenon as we could not observe any α -syn aggregation in control experiments where M83 substrates were maintained at 37°C, with or without vigorous shaking. It is known that, compared to its wild-type counterpart, human mutated A53T α -syn has an intrinsic tendency to aggregation (Flagmeier et al., 2016). Thus conceivably, *de novo* α -syn aggregation in the M83 substrate triggered by sonication-incubation cycles is favored by A53T-mutant human α -syn, similarly to what is observed naturally in this mouse model during aging of the animals, but here in a strongly accelerated manner and in a test tube.

Strikingly, we next demonstrate a PMCA seed-dependent aggregation of the endogenous mouse α -syn in both D and C57Bl/6-WT brain substrates. This seed-induced α -syn aggregation was obtained with seeds either generated *de novo* after PMCA of M83 brain, or derived from the brain of sick M83 mice. These results thus illustrate that PMCA allowed α -syn aggregates from M83 mice to replicate in C57Bl/6-WT mouse brain by crossing a transmission barrier between α -syn of different sequences in brain tissue. Immunoblot analysis using antibodies against human or mouse α -syn showed a preferential aggregation of human α -syn in the M83 brain substrate after PMCA, whereas human α -syn was poorly aggregated in the D brain substrate. Mouse α -syn however showed strong aggregation in the D and C57Bl/6-WT brain substrates after seeding with aggregated α -syn from M83 brain. One explanation for the preferential aggregation of mouse α -syn over human α -syn in the D substrate would be that mouse α -syn has a threonine residue at position 53, making it a more aggregation-prone substrate, similar to mutations in familial Parkinson's disease. An *in vitro* study examining why the aggregation of human α -syn was remarkably much slower than that of mouse α -syn indeed suggested that the presence of the A53 sequence in humans was

crucial and could even represent an evolutionary adaptation to minimize risk of Parkinson's disease (Kang et al., 2011).

Our observations of α -syn aggregation after a sonication-based PMCA procedure are not unprecedented (Fairfoul et al., 2016; Herva et al., 2014; Jung et al., 2017; Paciotti et al., 2018; Shahnawaz et al., 2017). Notwithstanding, recombinant α -syn diluted in saline solution was constantly used as a PMCA substrate in those previous PMCA studies, and α -syn aggregation was generally monitored using thioflavine-T fluorescence. A recent study however used Western blot detection and interestingly reported efficient seeding of PMCA reactions with brain or spinal cord extracts from two other transgenic mouse models expressing A53T mutant human α -syn (Jung et al., 2017), concurring with our current observations of a PMCA seeding activity in brain extracts of M83 mice. Our study also shows that this PMCA-induced α -syn aggregation could be easily monitored and quantified using an ELISA test that we previously described for the specific detection of pathological α -syn in M83 mice (Betemps et al., 2014; Sargent et al., 2017). Whereas ELISA immunoreactivity was overall found to be linked to the presence of SDS-resistant α -syn aggregates identified by Western blot in the PMCA samples, this was similarly found from C57Bl/6-WT brain, but only after seeding with seeds containing aggregated α -syn from M83 mice. This seeding was also required to identify SDS-resistant species by Western blot from C57Bl/6-WT brain substrate.

The precise nature of the M83-associated PMCA seeding activity in our experiments remains uncertain, but some lines of evidence suggest it is associated with the presence of α -syn aggregates. We consistently detected robust seeding activity in brains of sick M83 mice compared to healthy M83 mice. Importantly, this was equally seen in spontaneous *versus* accelerated illness of M83 mice, arguing against an age-dependent effect of brain material on

PMCA seeding activity. Finally, we observed that PMCA seeding activity within the same M83 brain is higher in regions mostly targeted by the α -syn pathology in this model.

Using serial PMCA rounds to increase our assay sensitivity led to the preclinical detection of α -syn seeding activity in some of the healthy (< 6 months old) M83 mouse brains analyzed. At these ages, our previous immunohistochemistry studies only showed cytoplasmic labelling of Ser129 phosphorylated α -syn in enteric neurons, but not in the brain (Bencsik et al., 2014). α -syn inclusions start to accumulate late in the M83 model, in which mice ultimately develop a clinical disease at very variable ages (Emmer et al., 2011; Giasson et al., 2002). In future studies it will be possible to examine the evolution of α -syn seeding activity at preclinical stages during the disease progression in M83 mice. It has been reported that all brain regions of M83 mice, including histologically unaffected regions, contained equivalent amounts of detergent-soluble SDS-resistant oligomers that delayed the formation of α -syn fibrils after seeding *in vitro* recombinant α -syn, contrary to oligomers derived from histologically affected brain regions (Tsika et al., 2010). This study also suggested that oligomers are formed at 4–6 months, before the formation of α -syn inclusions and onset of symptoms, consistent with the finding of a seeding activity in the brain of some of the healthy 3.5-5 month-old M83 mice in our study.

The bioassays for PMCA samples described here support the idea that *de novo* aggregated α -syn was a major contributor in the acceleration of the M83 disease after intracerebral inoculation. In control bioassays, we used inocula prepared from M83^{+/+} young brain substrate in standing and shaking conditions, as well as from PMCA-sonicated C57Bl/6-KO mice brain substrate. We found no evidence of disease acceleration after intracerebral inoculation of these samples to young M83^{+/+} mice. In contrast, mice inoculated with PMCA-sonicated M83 samples containing *de novo* α -syn aggregates developed characteristic clinical signs of experimental synucleinopathy after incubation periods of <120 days, which was

accompanied by accumulation of pathological α -syn within the brain. This prion-like acceleration of M83 disease was thus as efficient as previously described following intracerebral inoculations of brain homogenates from sick M83 mice or aggregated recombinant α -syn (Betemps et al., 2014; Luk et al., 2012; Mougenot et al., 2012). Interestingly, PMCA samples from hemizygous M83 mice also led to efficient transmission despite the fact that these mice are largely resistant to the development of spontaneous illness during normal aging in the absence of any additional challenge (Giasson et al., 2002; Prusiner et al., 2015). Also of note, α -syn from PMCA-sonicated M83 inocula was partially resistant to PK digestion, reminding the tight association of protease resistance with infectivity which is well known for the prion protein during prion diseases. Our bioassays of α -syn PMCA products were however limited to the examination of sonicated and unseeded brain homogenates in M83 mice. Whether these PMCA products can also transmit α -syn pathology to other hosts, including after seeded PMCA adaptation in different brain substrates, remains to be established.

The molecular events underlying the efficiency of PMCA are still uncertain. Since its initial description in 2001, the PMCA technology has been constantly evolving. Reproducibility and amplification performances of the technique have benefited considerably from a number of key modifications, including notably the addition of beads or polyanionic co-factors in PMCA reactions (Gonzalez-Montalban et al., 2011). In the present study, we similarly observed that Teflon beads strongly promote PMCA α -syn aggregation. It is generally believed that, through strong agitation, the sonication process fragments the growing polymers into smaller pieces, exposing more “free ends” of misfolded prion protein. Sonication also produces very high local temperature and pressure and involves a cavitation process, a repeated expansion and collapse of microbubbles splitting entrapped gaseous water molecules (sonolysis), resulting in the formation of reactive oxygen species such as H_2O_2 and

O₂ causing protein oxidation (Haigh & Drew, 2015). Sonication was already shown to promote formation of amyloid aggregates of a variety of structurally diverse proteins, with high stability against solubilization upon treatment by heat, detergent or reducing agent, and with the ability to further seed the aggregation of soluble protein (Stathopulos et al., 2004). Regarding α -syn specifically, its ultrasonication-induced fibrillation was reported some years ago using recombinant α -syn (Kim et al., 2007), and it was shown that after additional extensive ultrasonication in the absence of monomeric α -syn, the resulting fragments could serve as nucleation centers for the subsequent seed-dependent accelerated fibrillation under a quiescent incubation condition (Kim et al., 2007).

In conclusion, we have shown that a sonication-based PMCA procedure can be used as a new *in vitro* model of α -syn aggregation and seeded-propagation in a brain tissue matrix (**Figure 9**). A practical benefit of this system is the possibility to generate high amounts of brain-derived aggregated α -syn rapidly, and more generally, PMCA may offer unique opportunities of research, as in the field of prion diseases. A number of recent studies emphasized that a non-sonicated version of PMCA (called RT-QuIC) was promising for the diagnosis of synucleinopathies in humans (Fairfoul et al., 2016; Paciotti et al., 2018; Shahnawaz et al., 2017). The sonication-based PMCA approach for synucleinopathies in brain substrates may now help in deciphering the molecular basis of α -syn aggregation in a physiologically relevant environment, including the assessment of aggregation-inhibitory molecules with potential therapeutic applications (Ferreira & Caughey, 2018) or more basically, to better understand major unresolved questions in the field such as the possible existence of α -syn “strains” (Peelaerts et al., 2015; Peng et al., 2018; Prusiner et al., 2015; Watts et al., 2013).

Materials and Methods

Brain samples

PMCA substrates were prepared by homogenization of whole brains using a mechanical homogenizer (grinding balls, Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) to obtain a 5 to 10% homogenate (w/v) in converting buffer (PBS pH=7.2, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail). They were prepared from 2-month-old i) transgenic mice bred in our laboratory, overexpressing either the human A53T mutated α -syn under the prion promoter (M83 line)(Giasson et al., 2002) or the normal human α -syn under the PDGF- β promoter (D line)(Masliah et al., 2000) and ii) C57Bl/6 mice expressing (C57Bl/6-WT) or not (C57Bl/6-KO) mouse α -syn purchased from Janvier labs (Saint-Berthevin, France) and Envigo (Gannat, France) respectively (Specht & Schoepfer, 2001). M83 brain substrates were derived from homozygous (+/+) or hemizygous (+/-) mice obtained after breeding M83 homozygous mice with Bl/6;C3H mice, corresponding to the genetic background of the M83 mouse line (Giasson et al., 2002). In some experiments, brain homogenates obtained in High Salt Buffer (50 mM Tris-HCl pH 7.5, 750 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% phosphatase and protease inhibitor cocktails) were used as seeds in PMCA reactions. These seeds were prepared from whole brains of healthy ($\leq$ 6-month-old) or sick M83 mice, or from dissected brain regions. Sick M83 mice had developed the typical M83 disease either after normal aging ($\geq$ 1 year old)(n=3) or after intracerebral inoculations of brain homogenates from sick M83 mice or of fibrillar recombinant α -syn (Bousset et al., 2013), as previously described (Betemps et al., 2014). In one experiment PMCA was performed using seeds prepared from dissected brain regions of sick M83 mice, as previously described (Betemps et al., 2015).

428

429 **Protein Misfolding Cyclic Amplification (PMCA)**

430 PMCA procedure was previously described (Bougard et al., 2016). Briefly, 100µl of brain
 431 homogenates from healthy mice (M83^{+/+}, M83^{+/-}, D, C57Bl/6-WT and C57Bl/6-KO) were
 432 used directly in PMCA reactions at 5% or 10% in comparison with shaking (1000 rpm at
 433 37°C) and standing at 37°C. The same brain homogenates were also used as substrate either at
 434 5% (M83^{+/-}) or 10% (D, C57Bl/6-WT and C57Bl/6-KO) after mixing with 10µL of serially
 435 diluted brain homogenates (from 10⁻³ to 10⁻⁶) used as seeds. PMCA reactions were performed
 436 in PCR tubes containing three Teflon beads and submitted to amplification in a microplate
 437 sonicator (Q700; Qsonica). Each cycle is composed of an incubation step (29 min 40 s at
 438 37°C) and a sonication step (20 s at 140 W) for cycles ranging from 48 to 144 (1 round). For
 439 serial PMCA, amplified materials were diluted 1:10 in fresh PMCA substrate and submitted
 440 to a new PMCA round. To avoid any cross-contamination, experiments were carried out
 441 under strict quality-controlled PCR conditions.

442

443 **Western blot**

444 PMCA samples (10 µl) were mixed with 2 µl of TD4215 denaturing buffer (final
 445 concentration: 4% SDS, 2% β mercaptoethanol, 192 mM glycine, 25 mM Tris, 5% sucrose).
 446 Proteins were separated after heat denaturation (5 min at 100°C) on 12% TGX Stain Free
 447 FastCast acrylamide gels (#1610185, Biorad) loaded with 2 µl of the denatured PMCA
 448 samples per lane, then blotted onto PVDF membranes (Immobilon-P, Millipore). After
 449 migration, gels were activated for 1 min on Chemidoc MP apparatus (BioRad) and image
 450 analysis was done using ImageLab software (BioRad), to control the protein loads per lane
 451 (data not shown). α-syn was cross-linked to membranes using 0,4% paraformaldehyde diluted

in PBS for 30 minutes and unspecific binding sites were saturated by adding 5% milk for 1 h. α -syn was detected by either C-20R (1:10 000)(Cat #sc-7011-R Santa Cruz Biotechnology) rabbit polyclonal antibody, MJFR1 (1:1000) rabbit monoclonal antibody (Cat #ab138501, Abcam) or D37A6 (1:1000) rabbit monoclonal antibody (Cat #4179, Cell Signaling technology), diluted in PBST overnight at +4°C. Membranes were then incubated with stabilized goat anti rabbit HRP (1:1000) (Cat #32460, Interchim), incubated for 1 h at room temperature. HRP was then visualized with chemiluminescent substrate (Cat #34076, Supersignal WestDura, Thermo) and analyzed using the ChemiDoc system and ImageLab software (Bio-Rad). For some experiments, we performed pK digestion on PMCA samples, with pK concentration from 0,5 to 10 μ g/ml (final concentration). In the same manner, prior to migration on 12% TGX Stain Free FastCast acrylamide gels (#1610185, Biorad), PMCA digested products were mixed with 2 μ l of TD4215 denaturing buffer.

ELISA

For the analyses of the PMCA samples, 200 μ l of Superblock T20 (Thermo Scientific, Rockford, IL, USA) were loaded in each well of MaxiSorp™ plates (Thermo Scientific Nunc, Roskilde, Denmark) for 1 h at 25°C, under agitation at 150 rpm. After rinsing 5 times with PBST, the PMCA samples (2 μ l per well, adjusted to 100 μ l in PBST + 1% BSA) were then loaded and incubated for 2 h at 25°C, under agitation at 150 rpm. The plates were washed 5 times with PBST and primary antibodies C-20R (1:10 000) / MJFR1 (1:1000) / D37A6 (1:1000) in PBST + 1% BSA (Cat #15260037, Gibco, Thermo Fisher) were then incubated for 1 h at 25°C under agitation at 150rpm. The plates were washed another 5 times with PBST, and anti-rabbit IgG HRP conjugate (Cat #4010-05, Southern Biotech) was added (1:2000 dilution) in PBST + 1% BSA for 1h at 25°C. After washing the plates 5 times with

PBST, 100 μ l of 3,3',5,5'-tetramethylbenzidine solution (Cat #T0440, Sigma) were added to each well and plates were incubated for 15 min with shaking. The enzymatic reaction was stopped by adding 100 μ l of HCl 1M. Optical densities (ODs) were read at 450 nm by a microplate reader (CLARIOstar, BMG Labtech). OD values of blank wells, performed in parallel but without adding brain homogenates, were subtracted from OD values for each well. Following bioassays, the disease of M83 mice was confirmed by ELISA detection of Ser129 phosphorylated α -syn as previously described (Sargent et al., 2017), after α -syn capture with Syn303 antibody (1 μ g/ml)(Cat# 824301, BioLegend, Ozyme, St-Quentin-en-Yvelines, France) and detection with the rabbit polyclonal antibody against PSer129 α -syn (1/3000)(Cat# ab59264, Abcam, Cambridge, UK).

Bioassays

Bioassays were performed by stereotactic injection into the left striatum (anteroposterior (AP): +0.14, mediolateral (ML): +2, dorsoventral (DV): - 2.75) of 2 μ l of inoculum in 2 month-old M83^{+/+} mice. When such M83 mice are not inoculated, they develop characteristic motor symptoms between 8 and 16 months of life, beginning with reduced ambulation, balance disorders, partial paralysis of a hind leg and then progressing to prostration, difficulty in feeding, weight loss, hunched back and general paralysis (Giasson et al., 2002). In our experiments, the inoculated M83 mice were housed per group in enriched cages in a temperature-controlled room on a 12h light/dark cycle, and received water and food *ad libitum*, in our approved facilities (N° C69 387 0801), in accordance with EEC Directive 86/609/EEC and French decree No. 2013-118. The experimental protocol was authorized (N° 16-039) by the Comité d'éthique ComEth ANSES/ENVA/UPEC. Daily monitoring of the mice took place to detect any symptom of the M83 disease as described (Sargent et al., 2017),

including slow ambulation, balance disorders and partial paralysis of a hind limb. When mice develop these first symptoms or any inter-current disease not associated with M83 disease, we proceed to euthanasia by lethal intraperitoneal injection of dolethal. In the 3 experiments in which mice did not show clinical signs before the age of 9 months, corresponding to the inocula prepared from either unsonicated M83^{+/+} brain or from sonicated C57Bl/6-KO brain, surviving healthy mice were sacrificed at the age of ~ 14 months old.

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Additional information

Author contributions

S.N., J.V., M.B., D. Bougard, and T.B. designed research; S.N., J.V., M.B., C.M., and D. Betemps performed the experiments; S.N., J.V., M.B., C.M., D. Betemps, D. Bougard, and T.B. analyzed data; S.N. and T.B. wrote the paper.

Competing interests

The authors declare that no competing interests exist.

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Reference of the mouse	Inoculation	Status	Age at death (days)	α -syn aggregates detection by Western blot	
				M83 ^{+/-} substrate (1 round)	C57Bl/6-WT substrate (round 1/2)
1	-	Healthy	60	-	- / -
2	-	Healthy	90	-	- / -
3	-	Healthy	90	-	- / -
4	-	Healthy	90	-	- / -
5	-	Healthy	110	-	- / +
6	-	Healthy	110	-	- / +
7	-	Healthy	110	-	- / +
8	-	Healthy	146	-	- / +
9	-	Healthy	146	-	- / +
10	-	Healthy	146	-	- / -
11	-	Healthy	146	-	- / +
12	-	Healthy	180	-	- / -
13	-	Healthy	180	-	- / -
14	Fibrils	Sick	139	+	+ / +
15	Fibrils	Sick	139	+	+ / +
16	Sick M83 brain (1%)	Sick	141	+	+ / +
17	Sick M83 brain (5%)	Sick	142	+	- / +
18	Sick M83 brain (5%)	Sick	161	+	- / +
19	Fibrils	Sick	182	+	+ / +
20	-	Sick	365	+	+ / +
21	-	Sick	451	+	+ / +
22	-	Sick	457	+	+ / +

Table 1. α -syn PMCA seeding activities in a panel of brain samples from sick or healthy M83 mice. One or two PMCA rounds were performed in M83^{+/-} (5%, 96 cycles) or C57Bl/6-WT (10%, 144 cycles) brain substrate after seeding with whole brain homogenates of M83^{+/+} mice diluted at 10^{-4} or 10^{-3} for sick or healthy mice respectively. The sick M83^{+/+} mice were either old unchallenged mice (≥ 1 year old) or 3-6 months old mice after intracerebral inoculations of brain extracts of sick M83^{+/+} mice or of recombinant fibrils. The Western blot detection results represent the presence (+) or absence (-) of SDS-resistant α -syn aggregates detected with C-20R antibody, as shown in Figure 6.

Figure 1. PMCA triggers *de novo* α -syn aggregation in the M83 mouse brain. **(A)** Immunoblots of α -syn (C-20R antibody) in 10% brain homogenates from C57Bl/6-KO, C57Bl/6-WT, D, M83^{+/-} and M83^{+/+} mice, obtained after treatment by 144 PMCA cycles or maintained in shaking and standing conditions, compared to the initial frozen brain homogenates. Abundant α -syn aggregates are specifically detected after PMCA in the M83 substrate. **(B)** PMCA α -syn aggregation kinetics in M83^{+/-} and M83^{+/+} substrates at two different normal brain homogenate (NBH) concentrations (5% *versus* 10%) and as a function of sonication-incubation cycles number (48, 96, 144). **(C)** Immunoblot of β -syn (Ab7611 antibody) in 10% brain homogenates from C57Bl/6-KO, C57Bl/6-WT, D, M83^{+/-} and M83^{+/+} mice after treatment by 144 PMCA cycles. Rec: human recombinant α -syn control. Bars to the left of each panel indicate the ~ 20, 30, 40, 50 and 60 kDa molecular weight markers.

Figure 2. PMCA products from M83 mouse brain contain partially proteinase K-resistant α-syn species. Immunoblots of α-syn (C-20R antibody) in 10% brain homogenates from M83^{+/-}, D and C57Bl/6-WT mice treated or not by 144 PMCA cycles, after proteinase K digestion for 30 min at 0, 0.5, 1, 2.5, 5 and 10 μg/ml final concentrations. M: molecular weight marker. Rec: human recombinant α-syn control. Bars to the left of each panel indicate the ~ 20, 30 and 40 kDa molecular weight markers.

Figure 3. *De novo* PMCA-generated α -syn aggregates from M83 mice seed the aggregation of α -syn in PMCA reactions. **(A-B)** Immunoblots of α -syn (C-20R antibody) in PMCA samples after seeding with serial 10-fold dilutions (10^{-3} to 10^{-6}) of a 144 cycle PMCA product from M83^{+/-} mice containing *de novo* α -syn aggregates, in comparison with unseeded normal brain homogenate substrate treated by PMCA (NBH) or after seeding with unsonicated M83^{+/-} brain at dilution 10^{-3} (CTL). 144 PMCA cycles in 10% brain homogenates were performed for the C57Bl/6-KO, C57Bl/6-WT and D substrates, or 96 PMCA cycles in 5% brain homogenates for the M83^{+/-} substrate. Rec: human recombinant α -syn control. Bars to the left of each panel indicate the ~ 20, 30, 40, 50 and 60 kDa molecular weight markers. **(C-D)** ELISA immunoreactivity after loading of the same PMCA samples on MaxiSorp™ plates, followed by detection of captured α -syn using C-20R antibody. Three repetitions of the ELISA test were performed.

Figure 4. Distinct aggregation behavior of human and mouse α -syn proteins after seeded-PMCA in the different brain substrates. **(A-C)** Immunoblots of α -syn in PMCA samples obtained after seeding the four mouse brain substrates with a PMCA product (dilutions 10^{-3} and 10^{-4}) produced in M83^{+/-} brain as shown in Figure 1A. 144 PMCA cycles in 10% brain homogenates were performed for the C57Bl/6-KO, C57Bl/6-WT and D substrates, or 96 PMCA cycles in 5% brain homogenates for the M83^{+/-} substrate. The immunoblot membranes were revealed by MJFR1 or D37A6 antibodies specific for the human or mouse α -syn respectively, or by C-20R antibody which recognizes both human and mouse α -syn. Rec: human recombinant α -syn control. Bars to the left of each panel indicate the ~ 20, 30, 40, 50 and 60 kDa molecular weight markers. **(D)** PMCA products were loaded on MaxiSorp™ plates and captured α -syn was detected using C-20-R, MJFR1 or D37A6 antibodies. Three repetitions of the ELISA test were performed.

Figure 5. Detection of α -syn PMCA seeding activity in the brain of sick M83 mice. **(A)** Western blot analysis of PMCA products obtained after seeding with serial 10-fold dilutions (10^{-3} to 10^{-6}) of a brain homogenate from a sick M83^{+/+} mouse, in comparison with unseeded normal brain homogenate substrate treated by PMCA (NBH) or after seeding with unsonicated healthy M83^{+/+} brain at dilution 10^{-3} (CTL). 144 PMCA cycles in 10% brain homogenates were performed for the C57Bl/6-KO, C57Bl/6-WT and D substrates, or 96 PMCA cycles in 5% brain homogenates for the M83^{+/+} substrate. The sick M83 mouse was a 170 days old mice that had been intracerebrally inoculated at 8 weeks old with brain homogenate of sick M83 mouse and the healthy mouse was a 60 days old unchallenged mouse. **(B)** Immunoblots of α -syn (C-20R antibody) in PMCA samples obtained after seeding the C57Bl/6-WT brain substrate (10%, 144 cycles) with a whole brain homogenate (dilutions 10^{-3} to 10^{-10}) from a sick M83 mouse that died with typical clinical signs of the disease, in comparison with unseeded normal brain homogenate (NBH). One or two PMCA rounds were performed. Rec: human recombinant α -syn control. Bars to the left of each panel indicate the ~ 20, 30, 40, 50 and 60 kDa molecular weight markers.

Figure 6. PMCA seeding activity in a panel of brain samples from sick or healthy M83 mice. **(A)** Immunoblots of α -syn (C-20R antibody) in PMCA products obtained after seeding C57Bl/6-WT (10%, 144 cycles) substrate with brain samples from healthy M83^{+/+} mice at dilution 10^{-3} . One or two PMCA rounds were performed. **(B)** Immunoblots of α -syn (C-20R antibody) in PMCA products obtained after seeding M83^{+/+} (5%, 96 cycles) or C57Bl/6-WT (10%, 144 cycles) substrates with brain samples from sick M83^{+/+} mice at dilution 10^{-4} . The figures below each of the panels indicate the reference of the mouse as listed in Table 1. Rec: human recombinant α -syn control. Bars to the left of each panel indicate the ~ 20, 30, 40, 50 and 60 kDa molecular weight markers. **(C)** ELISA detection of Ser129 phosphorylated α -syn in the same panel of brain samples from healthy or sick M83^{+/+} mice. ELISA detection was performed using Syn303 as a capture antibody and rabbit polyclonal antibody against Ser129 phosphorylated α -syn (Abcam Cat# ab59264) for detection. Two quantities of brain homogenates were examined, including 0.2% (as described by Sargent et al., 2017) and 0.01% homogenates, this last concentration corresponding to a 10^{-4} dilution of seeds as examined in PMCA experiments.

Figure 7. PMCA seeding activity within the same M83 brain is higher in regions mostly targeted by the α -syn pathology. **(A)** Comparison of PMCA seeding activities in different brain regions of two sick M83^{+/+} mice sacrificed with typical clinical signs, at the age of 573 (sick M83 #1) or 670 (sick M83 #2) days old. PMCA was performed using the C57Bl/6-WT brain substrate (10%, 144 cycles) after seeding with a 10^{-4} dilution of each of the following brain regions: olfactory bulb (OB), cerebral cortex (CTX), hippocampus (HIP), striatum (STR), midbrain (MB), cerebellum (CB) and brain stem (BS). Bars to the left of each panel indicate the ~ 20, 30, 40, 50 and 60 kDa molecular weight markers. **(B)** ELISA detection of Ser129 phosphorylated α -syn in the same dissected brain regions from sick M83 #1 and #2 mice. Two quantities of brain homogenates were examined, including 0.2% (as described by Sargent et al., 2017) and 0.01% homogenates, this last concentration corresponding to the 10^{-4} dilution of seeds as examined by PMCA.

Figure 8. Survival of M83^{+/-} mice after intracerebral inoculation of PMCA products. The age at death is indicated by the vertical axis for the 5 experiments with inocula prepared from M83^{+/-}, M83^{+/-} or C57Bl/6-KO brain homogenates (10%) which had been treated by PMCA for a single round (144 cycles) or, in the case of the M83^{+/-} brain substrate, incubated without sonication in shaking or in standing conditions. The mean and standard deviations of the ages at death are indicated within the graph for the two groups from which all the mice had died before the end of the protocol at 400 days. Mice with the typical clinical signs of M83 disease are illustrated as circles and mice that were found dead without previously observing clinical signs as triangles; mice that were sacrificed after the end of the protocol, without detectable clinical signs are shown as squares. Mice from which brain samples were found positive by ELISA for Ser129 phosphorylated α -syn are shown in red and those that are negative in green; those that could not be analyzed because of unavailable frozen samples are shown in black.

Figure 9. Schematic representation of PMCA generation and seeded propagation of lethal α -syn aggregates. PMCA provokes spontaneous, sonication-dependant, α -syn aggregation from young M83 brain homogenate. *De novo* generated α -syn aggregation from M83 brain propagates in a wild-type mouse brain substrate after seeded-PMCA and accelerates α -syn neuropathology and disease after intracerebral inoculation to young M83 mice. These findings corroborate the prion-like character of aggregated α -syn. PMCA emerges as a potential *in vitro* model of α -syn aggregation phenomenon in a biological complex matrix.