Video Article

Detection of Disease-associated α-synuclein by Enhanced ELISA in the Brain of Transgenic Mice Overexpressing Human A53T Mutated α-synuclein

Dominique Bétemps[1], Jérémy Verchère[1], Anne-Laure Mougenot[2], Ingolf Lachmann[3], Eric Morignat[4], Emilie Antier[5], Latifa Lakhdar[5], Stéphane Legastelois[2], Thierry Baron[1]

[1]Neurodegenerative Diseases Unit, ANSES - French Agency for Food, Environmental and Occupational Health & Safety

[2]Indicia Production

[3]AJ Roboscreen GmbH

[4]Epidemiology Unit, ANSES - French Agency for Food, Environmental and Occupational Health & Safety

[5]Experimental Facilities, ANSES - French Agency for Food, Environmental and Occupational Health & Safety

Correspondence to: Thierry Baron at Thierry.BARON@anses.fr

URL: https://www.jove.com/video/52752
DOI: doi:10.3791/52752

Keywords: Medicine, Issue 99, Parkinson's, dementia, alpha-synuclein, prion, mouse, transgenic, ELISA

Date Published: 5/30/2015

Citation: Bétemps, D., Verchère, J., Mougenot, A.L., Lachmann, I., Morignat, E., Antier, E., Lakhdar, L., Legastelois, S., Baron, T. Detection of Disease-associated α-synuclein by Enhanced ELISA in the Brain of Transgenic Mice Overexpressing Human A53T Mutated α-synuclein. *J. Vis. Exp.* (99), e52752, doi:10.3791/52752 (2015).

Abstract

In addition to established methods like Western blot, new methods are needed to quickly and easily quantify disease-associated α-synuclein (αS^D) in experimental models of synucleopathies. A transgenic mouse line (M83) over-expressing the human A53T αS and spontaneously developing a dramatic clinical phenotype between eight and 22 months of age, characterized by symptoms including weight loss, prostration, and severe motor impairment, was used in this study. For molecular analyses of αS^D (disease-associated αS) in these mice, an ELISA was designed to specifically quantify αS^D in sick mice. Analysis of the central nervous system in this mouse model showed the presence of αS^D mainly in the caudal brain regions and the spinal cord. There were no differences in αS^D distribution between different experimental conditions leading to clinical disease, *i.e.,* in uninoculated and normally aging transgenic mice and in mice inoculated with brain extracts from sick mice. The specific detection of αS^D immunoreactivity using an antibody against Ser129 phosphorylated αS by ELISA essentially correlated with that obtained by Western blot and immunohistochemistry. Unexpectedly, similar results were observed with several other antibodies against the C-terminal part of αS. The propagation of αS^D, suggesting the involvement of a “prion-like” mechanism, can thus be easily monitored and quantified in this mouse model using an ELISA approach.

Video Link

The video component of this article can be found at https://www.jove.com/video/52752/

Introduction

Most current methods for detecting disease-associated α-synuclein (αS^D) in experimental models of Parkinson's disease (PD), such as immunohistochemistry or Western blot, are time-consuming and not quantitative. This neurodegenerative disease is characterized by alpha-synuclein aggregation mainly in the form of inclusions containing an aggregated form of the normally soluble presynaptic protein αS[1,2] (Lewy bodies and Lewy neurites). Normally only marginally phosphorylated, αS is hyperphosphorylated at its serine 129 residue in these inclusions[3] and can be monitored by antibodies specifically directed against Ser129 phosphorylated αS, thus providing a reliable marker of the pathology.

Recent research suggests that a “prion-like” mechanism could be involved in the propagation of αS aggregation within the nervous system of an affected patient[4,5]. These studies reported the acceleration of a synucleinopathy by inoculating brain extracts containing αS^D into a transgenic mouse model (M83) expressing an A53T mutated human αS protein associated with a severe motor impairment occurring as the mice age[6]. In the same manner, intra-cerebral inoculation of aggregated recombinant αS in the same M83 mouse model confirmed the acceleration of aggregation[5]. The induction of deposits of phosphorylated αS has also been reported after inoculation of C57Bl/6 wild-type mice with either fibrillar recombinant αS or brain extracts from human DLB patients[7,8]. Sacino *et al.*[9] recently pointed out that after injection of fibrillar human αS, a widespread and progressive cerebral αS inclusion formation could be induced in M83 mice, but not in E46K transgenic mice or non-transgenic mice in which induced αS inclusions were transient, and mainly restricted to the site of injection. Recent studies on monkeys confirmed propagation of αS aggregates after inoculation of PD-derived extracts in species closer to humans[10].

The link between αS alterations and Parkinson’s disease suggest that αS^D is a potential biomarker for Parkinson’s disease[11]. A recent study showed the detection of oligomeric soluble aggregates of α-synuclein in human cerebro-spinal fluid (CSF) and plasma as a potential biomarker for Parkinson’s disease based on a conventional sandwich system ELISA using the same antibody to capture and detect αS[12]. Based on the same method, multimeric proteins were recognized in biological samples, including the brain, because there are multiple copies of epitopes present in the assembled forms[13]. Very recently, pathological αS in the CSF of patients with a proven Lewy body pathology was detected using

both an ELISA kit with a highly specific antibody against αS^D (5G4) and an immunoprecipitation assay[14]. These methods could differentiate patients with PD/DLB from other types of dementia.

The “prion-like” propagation of αS aggregation was further studied in transgenic mouse model M83 using an ELISA approach that was designed to specifically identify αS^{D15}. In this study, we report the detailed ELISA protocol used to quantitatively detect αS^D in sick mice (whether or not inoculated with αS^D from sick M83 mice) and more especially in the brain regions specifically targeted by the pathological process in this M83 transgenic mouse model[4].

Protocol

All the procedures and protocols involving animals were in accordance with EC Directive 86/609/EEC and ratified by ComEth, the French national committee for consideration of ethics in animal experimentation (protocol 11-0043). The animals were housed and cared for in ANSES's approved experimental facilities in Lyon (approval B 69387 0801).

1. Preparation of Mice

1. Euthanize mice by an intraperitoneal injection of lethal dose of sodium pentobarbital.
2. Retrieve the whole brain from the mouse skull and place it in a 35 mm plastic Petri dish on ice until extraction.
3. Extract the cervical spinal cord.
   NOTE: Extract αS either from one of the brain halves after sagittal sectioning or from dissected mouse brains, available after the experiments listed in **Table 1**.

Experiment	Mice Inoculum (brain equivalent)	Survival period (d.p.i.)	Median/maximal survival (days old)	αS^d detection by ELISA /WB/IHC
1	Uninoculated mice	441 ± 166	419/736	8/8
2	Inoculated mice (0.2 mg)	150 ± 52	140/241	9/9

Table 1. List of experiments performed on M83 mice. Inoculations were performed at 6 weeks for experiment 2 in the striato-cortical area with 20 µl of a brain homogenate of a sick mouse (1% wt/vol in glucose 5%), after anesthesia of 6 weeks old homozygous M83 mice by 3% isoflurane inhalation. d.p.i.: days post inoculation.

2. αS Extraction from Brain Halves

1. Sagittally cut the brain to obtain two halves. Weigh each half in a ribolysis tube containing grinding balls.
2. Prepare High Salt (HS) buffer containing 50 mM Tris-HCl, pH 7.5, 750 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% phosphatase and protease inhibitor cocktails. Add High Salt buffer to the brain halves to obtain 20% (weight/volume) homogenates.
3. Prepare samples from the brain halves using a mechanical homogenizer at 6.0 m/s for 23 sec twice. After the first 23 sec homogenization, place the tubes containing the homogenates on ice for 2 min before the second 23-sec cycle.
4. Centrifuge the samples at 1,000 x g for 5 min to eliminate unground brain fragments. Recover the supernatants, divide into 200 µl aliquots and keep them at -80 °C for subsequent ELISA analysis.

3. αS Extraction from Dissected Brain Regions

1. Dissect a whole brain in a 35 mm plastic Petri dish on ice with a low power magnifier (8X magnification) using two forcipes whose ends are kept together except when dissecting the hippocampus. Do not exceed 10 min to preserve brain integrity. Place the brain right side up and retrieve the following brain regions in this order:
   1. Separate one of the two olfactory bulbs using forceps placed just behind the bulb. Detach it from the brain by a downwards movement. Repeat this operation for the second bulb.
   2. Gently wedge the forceps in between the two cortexes and move it forwards to facilitate dissociation of the two cortexes. Keeping the brain in place with one forceps, use another to separate the cortex from the hippocampus.
   3. Position the forceps 2 mm below the cortex. Maintain a gentle pressure on the forceps until the top of the hippocampus is visible. Peel off the first part of the cortex, and repeat with the second part. Use the forceps to separate the two cortexes starting at the hippocampus and moving towards the front of the brain.
   4. Position the open forceps around one of the hippocampi. Close the forceps at the bottom of the hippocampus then gently remove it, recovering as much as possible. Repeat the procedure for the second hippocampus.
   5. Position the open forceps below one of the striata and gently separate it from the brain. Use the forceps to remove any remaining cortex from the striatum. Repeat this procedure for the second striatum.
   6. Use the forceps to gently depress by 2 mm the contour of the cerebellum to facilitate separation of the cerebellum from the brain. Place the forceps just behind the cerebellum and remove it by moving the forceps forward.
   7. Use the wide part of the forceps to raise the mesencephalon in order to clearly see where it joins the brain stem. Make a vertical incision at the junction then remove the brain stem.
   8. Position the forceps behind the mesencephalon, which is composed of four rounded structures. Incise vertically until the mesencephalon has been completely separated from the remaining brain.
2. Prepare homogenates of variable % (weight/volume) in HS buffer, depending on the quantity of available tissues, *i.e.,* 5% homogenate for a weight between 10 and 30 mg, 10% for a weight between 30 and 80 mg, and 20% for a weight above 80 mg.

1. Add an adequate volume of HS buffer to the dissected brain regions to obtain the expected % of homogenate.
2. Vortex and check that the tissues are fully immersed in the HS buffer.
3. Homogenize samples prepared from the dissected brain regions or the cervical spinal cord with a tissue grinder composed of a borosilicate glass tube and two pestles, A and B.
4. Pour each brain region to be crushed directly into the tube. Insert pestle A into the tube and retract it. Repeat this movement about ten times to dissociate the tissue. Then use pestle B to continue grinding the tissue with a further 20 movements. Transfer the homogenates into a 1.5 ml tube with a 1 ml transfer pipette.

3. Centrifuge the samples at 1,000 x g for 5 min at 4 °C to eliminate any unground brain fragments. Retrieve the supernatants, divide them up into 200 µl aliquots and keep them at -80 °C for subsequent ELISA analysis.

4. Detection of αS by ELISA

1. Dilute the coating antibodies to 0.01 ng/ml. Use either anti-αS rabbit polyclonal or monoclonal clone 42 antibody in 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 9.6).
2. Coat the 96-well microplates with 100 µl per well of this coating solution, and leave at 4 °C O/N. Use the anti-αS rabbit polyclonal antibody in the coating solution for ELISAs using detection antibodies syn514, clone 42, LB509, AS11, 4D6 or 8A5. Use the anti-αS monoclonal antibody clone 42 as a coating solution in combination with the anti-pSer129 αS detection antibody.
   NOTE: If necessary, the plates may be kept at 4 °C for one week before the ELISA is performed.
3. Use a plate washer to wash the plates five times with 300 µl of phosphate-buffered saline with 0.05% Tween 20 (PBST) per well. From this step onward, incubation is at RT.
4. Add 200 µl of T20 PBS blocking buffer per well. Shake for 1 hr at 150 rpm. Wash the plates five times with PBST.
5. Dilute the brain homogenates (dilution 1:100 of the 20% homogenates, 1:50 of the 10% homogenates and 1:25 of the 5% homogenates in PBST BSA 1%), and add 100 µl to each well. Then incubate for 2 hr while shaking at 150 rpm. Wash the plates five times with PBST.
6. Add the different αS detection antibodies in PBST with BSA 1% at the dilutions mentioned in the Materials List. Incubate for 1 hr at 150 rpm. Wash the plates five times with PBST.
7. Add either anti-mouse or anti-rabbit IgG HRP conjugates diluted 1:8,000 in PBST supplemented with BSA 1% for 1 hr while shaking at 150 rpm. Wash the plates five times with PBST.
8. Add 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) solution to each well and incubate for 15 min in the dark while shaking at 150 rpm.
9. Stop the reaction by adding 100 µl of 1 N HCl per well then measure the absorbance at 450 nm with the microplate reader.
10. For data analysis, subtract the OD value obtained in a well with all the reagents except any mouse brain samples (blank well) from the OD values measured for each of the analyzed samples.

5. Epitope Mapping

1. Perform epitope mapping according to the method described by Osman[16]. Briefly, spot peptides of the human α-synuclein sequence containing 12 amino acids on nitrocellulose with 10 overlapping amino acids.
2. Block with 50 mM Tris/150 mM NaCl buffer pH 10 containing 0.05% Tween 20 and 5% milk powder. Incubate the antibody in blocking solution at a concentration of 2 µg antibody per ml at 2-10 °C O/N.
3. Wash the membrane three times using 50 mM Tris/150 mM NaCl buffer pH 10 containing 0.05% Tween 20. Incubate with the goat anti-mouse IgG HRP conjugate. Wash the membrane another five times using the same buffer then stain using a Western blot TMB staining kit.

6. Statistical Analysis

1. Use the R software and nlme package to perform mixed-effects regressions to model OD. For each comparison, perform a mixed effect regression model. Use a fixed effect to distinguish symptomatic from asymptomatic groups.
2. Use a random effect to reflect the variability of repetitions for a given mouse. Check homoscedasticity by examining the residuals and if needed, use the variance functions to model the variance structure of within-group errors in keeping with Pinheiro and Bates[17]. Set 0.05 as the significance threshold of P.

Representative Results

In this study, the ELISAs used specifically identified disease-associated αS ($αS^D$) in brain homogenates prepared in a High Salt buffer from sick M83 mice. Using an antibody specifically recognizing pSer129 αS ($p = 0.0074$), the ELISA readily distinguishes old, sick mice (> 8 months old) from young (2-5 months old), healthy M83 mice (**Figure 1**). Several other antibodies showed similarly high signals (> 0.6 OD) only in brain homogenates from sick mice. This is the case for 4D6 ($p = 0.01$), LB509 ($p = 0.0047$) and 8A5 ($p < 0.001$) against different sequences of the C-terminal part of the protein (124-134, 115-122, and 129-140 respectively) and, to a much lesser extent, syn514 against the N-terminal end (2-12) of the protein ($p = 0.0003$). Furthermore, the AS11 monoclonal antibody produced in our laboratory against human fibrillar recombinant αS[18] after immunization of C57BL/6S (B6 αS-null)[19] mice with a deletion of the α-syn locus, showed similarly high signals ($p < 0.01$) only in brain homogenates from sick mice. This antibody has now been shown to recognize the amino-acid sequence (P)VDPDNEAY(E) of αS, corresponding to the 118-125 sequence of human α-synuclein.

In contrast, under these experimental conditions, analyses of brain homogenates with the clone 42 detection antibody directed against a central region of α-synuclein (91-96 sequence), showed a very low signal for both sick and healthy M83 mice. It could not distinguish the two populations of mice ($p = 0.1158$). Young M83 mice nevertheless showed a higher immunoreactivity than did non transgenic B6C3H mice (M83 genetic background line). The contrast was even greater with C57BL/6S (B6 αS-null) mice, which have a deleted α-syn locus. This suggests that the slight immunoreactivity in M83 mice corresponds to low signal detection of normal αS. The analytical sensitivity of this ELISA was assessed in

comparison to a previously described Western blot method[4] for the detection of insoluble Ser129 phosphorylated α-synuclein, by examining, using both ELISA and Western blot methods, serial dilutions of brain homogenates prepared from the same sick M83 mouse brain (**Figure 2**). Approximate 10 µg brain equivalents were sufficient to obtain a positive ELISA signal for the brain homogenate from this sick mouse, with both LB509 and PSer129 antibodies. On the contrary, at least 200 µg brain equivalents were needed to detect pSer129 αS in the sarkosyl-insoluble fraction analyzed by Western blot using the same PSer129 antibody[15]. This indicates that the ELISA has an analytical sensitivity some 20 times that of the Western blot method.

In sick and old M83 mice (**Figure 3A**), brain homogenates from the mesencephalon, brain stem and spinal cord showed marked immunoreactivity with both LB509 and PSer129 antibodies in the ELISA. However, no detectable immunoreactivity was observed in the other brain regions, including the olfactory bulb, cerebral cortex, striatum, hippocampus, thalamus and hypothalamus. The ELISA gave a faint signal for the cerebellum. The immunoreactivity in the different brain regions of M83 mice developing an accelerated clinical disease following the injection of a brain extract from a sick M83 mouse[4] (**Figure 3B**) was indistinguishable from that seen in aged (> 8 months old) uninoculated M83 mice. These results are fully consistent with those obtained by Western blot (**Figure 3C**) and immunohistochemistry[15], showing a much greater deposition of Ser129 α-synuclein in the caudal regions of the brain and spinal cord.

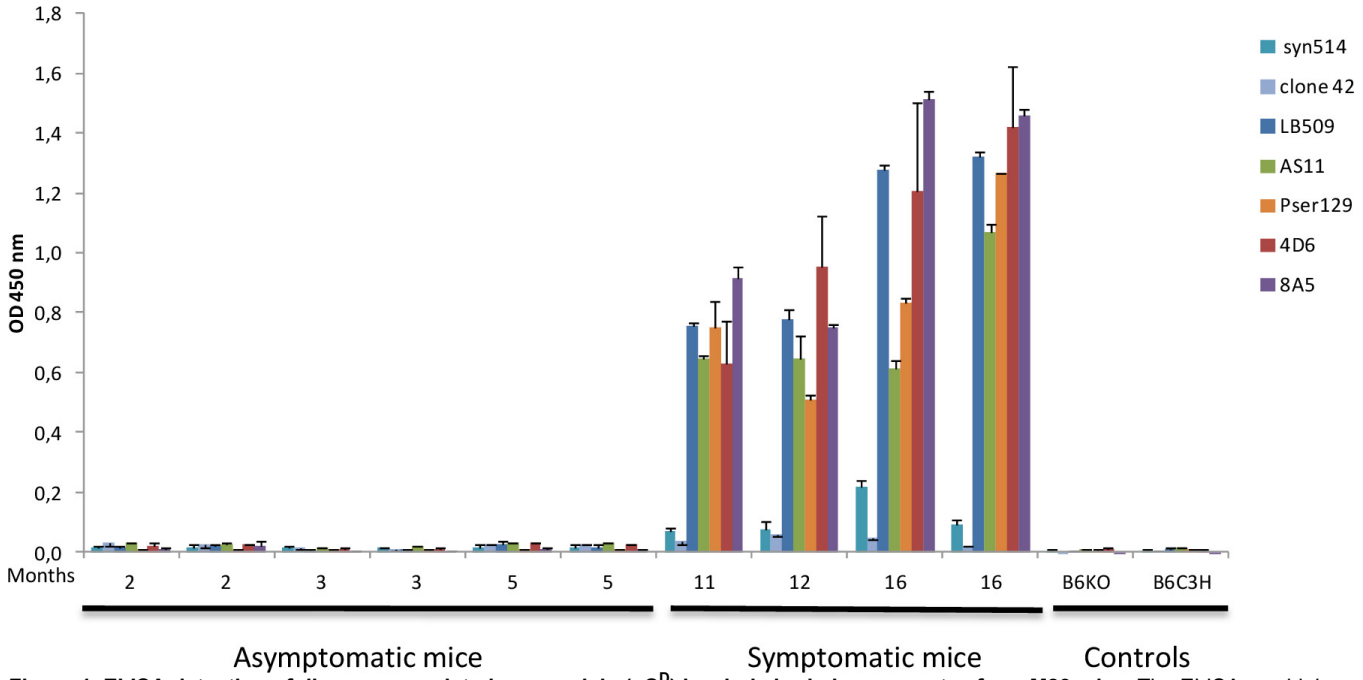
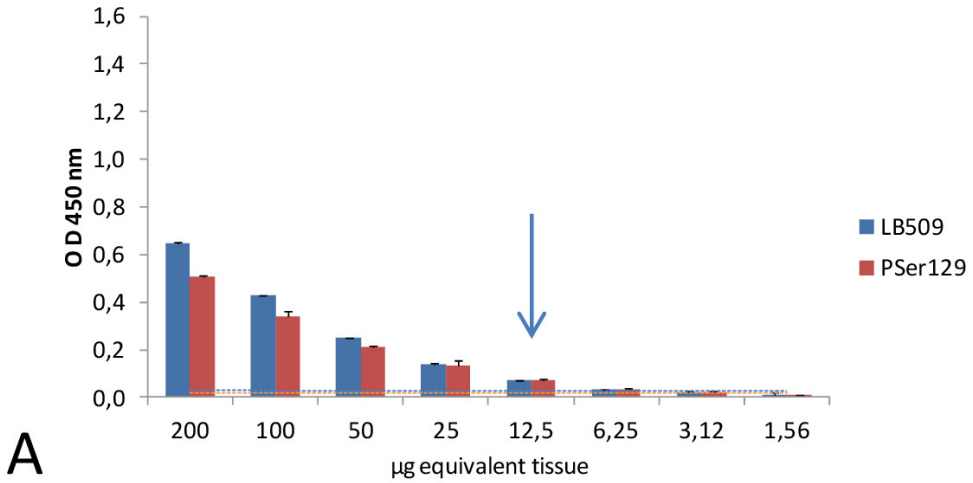


Figure 1. ELISA detection of disease-associated α-synuclein (αS^D) in whole brain homogenates from M83 mice. The ELISA combining rabbit anti-αS polyclonal (coating) with syn514, LB509, AS11, 4D6, 8A5 (detection) or clone 42 (coating) with anti-pSer129 αS (PSer129) (detection) antibodies distinguish sick mice from healthy M83 mice, whereas ELISA with anti-αS rabbit polyclonal (coating)/clone42 (detection) does not. The six sick, old M83 mice aged from 11 to 16 months showed signs of immunoreactivity with the anti α-syn antibodies (except clone 42 antibody). This was not the case for either the six young, healthy mice aged from 2 to 5 months old, or with the additional controls including B6C3H (genetic background of M83 mice) or B6 αS-null mice. The error bars represent S.D. Modified from Bétemps[15].



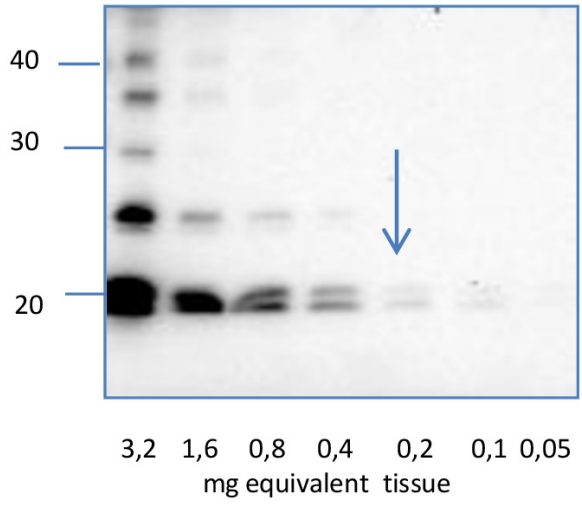


Figure 2. Comparison of the analytical sensitivity of detection of αS^D by ELISA and Western blot. **A.** A positive signal was obtained with ELISA for 12.5 µg brain equivalents with both LB509 and PSer129 antibodies from two-fold dilutions of brain homogenates from a sick mouse. The estimated cut-off level for the discrimination of sick and healthy mice, corresponding to the means obtained from samples of healthy M83 mice during three to six repeats of ELISA measures + 3 standard deviations, were 0.030 and 0.020 for LB509 and PSer129 detection antibodies respectively. They are shown as a line in the same color as that used for each of the antibodies. **B.** By Western blot analysis of the insoluble fractions obtained after ultracentrifugation in the presence of sarkosyl, 200 µg brain equivalents were needed to detect αS^D with the same PSer129 antibody. Molecular weight markers (in kDa) are indicated on the left of the blot. Reprinted with permission from Bétemps[15].

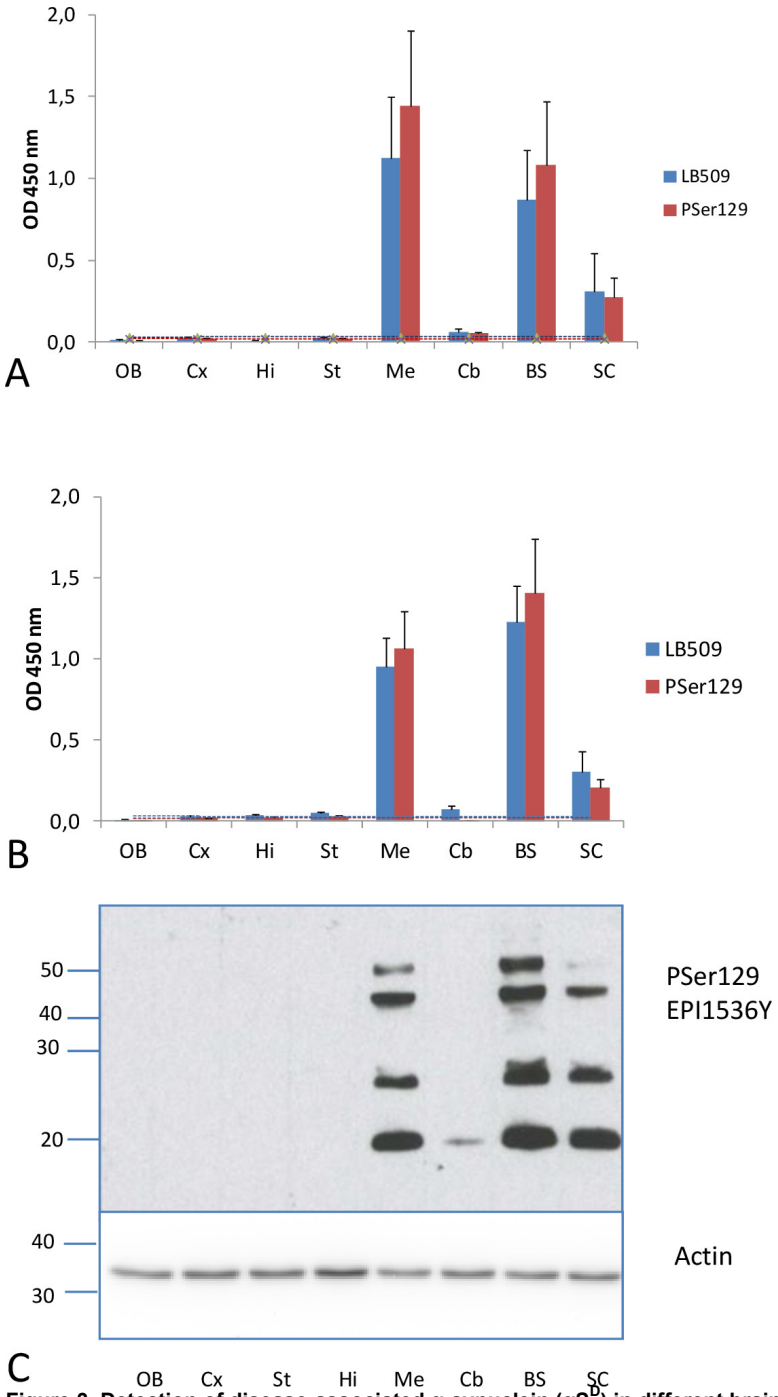


Figure 3. **Detection of disease-associated α-synuclein (αS^D) in different brain regions of M83 mice by ELISA and Western blot**. ELISA identified αS^D with PSer129 or LB509 detection antibodies in M83 mice during normal aging (**A**) (n = 1, 4 repeats, mean ± S.D.) or after inoculation of 6 week-old M83 mice with a brain homogenate from a sick M83 (**B**) (n = 1, 4 repeats, mean ± SD). (**C**) αS^D was identified by Western blot with the PSer129 detection antibody EP1536Y in the same neuro-anatomical regions in mice inoculated with a brain homogenate from a sick M83 mouse. Molecular weight markers (in kDa) are indicated on the left of panel C. Equal amounts of total proteins were used in each line for equivalent loading on gel. The following eight neuro-anatomical regions from sick M83 mice were tested: OB: olfactive bulb, Cx: cerebral cortex, Hi: hippocampus, St: striatum, Me: mesencephalon, Cb: cerebellum, BS: brain stem, SC: cervical spinal cord. Modified from Bétemps[15].

Discussion

The use of an ELISA was demonstrated to specifically detect αS^D directly from mouse brain homogenates during the disease in the M83 transgenic mouse model. Indeed, this ELISA could readily distinguish sick M83 mice from healthy M83 mice using only whole brain homogenates in High Salt buffer.

The most critical steps for successful results using this ELISA are: correctly dissecting the different regions of the mouse brains by developing the necessary manual dexterity to prevent damage during dissection; performing sample dilutions exclusively in HS buffer; and the choice of antibodies, because not all antibodies will work in an ELISA format.

Some variability in αS^D levels was nevertheless apparent between different mice. These results are equivalent to the Western blot detection of the typical αS^D pattern only detected in sick M83 mice by examination of the sarkosyl-insoluble fractions after detection with an antibody against Ser129 phosphorylated αS[15]. This demonstrates that ELISA immunoreactivity corresponds to the specific detection of αS^D.

In agreement with previous Western blot analyses[15], this ELISA detected immunoreactivity after dissection of the brain of sick M83 mice in the caudal parts of the brain, *i.e.,* the mesencephalon and the brain stem, as well as in the cervical spinal cord. No immunoreactivity was detected in the olfactory bulb, cerebral cortex, striatum or the hippocampus, consistent with previous data indicating that the hippocampus was spared by the pathological process[6], unless at very low levels in the case of mice that had been inoculated by fibrillar alpha-synuclein into the hippocampus[15]. The distribution of αS^D thus appeared remarkably uniform overall, whatever the experimental conditions, which included either normal aging of mice or accelerated development of the disease after intra-cerebral inoculation of brain homogenates from sick M83 mice.

Furthermore, assay performance is better than the previously described Western blot method. The analytical sensitivity of the ELISA appeared higher, as shown by the limits of detection obtained in this test using serial dilutions of a brain homogenate from a sick M83 mouse (12.5 µg for ELISA *vs* 200 µg for the Western blot assay). The sensitivity of the ELISA however remained lower than that of immunohistochemical detection, which detects αS^D in individual cells and in frontal brain regions such as the cerebral cortex[15]. The sensitivity of the test could nevertheless be further improved by using different antibodies and/or optimized detection systems, such as chemiluminescent detection as previously described[13].

It is important to note that several other antibodies recognizing other forms of the protein, and notably a non-phosphorylated form (detected with the 4D6 monoclonal antibody[20]) gave similar results using this ELISA, in addition to the monoclonal antibody specifically recognizing pSer129 αS. This ELISA approach also revealed immunoreactivity with antibodies recognizing specifically human αS (LB509) and, to a much lower extent, mouse αS(D37A6) in the same sick animals and/or brain regions of sick mice[15]. On the other hand, no immunoreactivity was observed by the ELISA analysis of sick M83 mice using the clone 42 antibody against a central region of αS (91-96)[21,22], corresponding to an epitope which could be cryptic under these ELISA conditions. This ELISA format could detect in M83 mouse brains both Ser129 phosphorylated αS, as observed by Foulds[11] in human plasma, and non-phosphorylated, possibly oligomeric forms, as described in human plasma and cerebrospinal fluid[12,14]. Unlike previously published ELISAs, our ELISA format neither used the same antibody in capture and detection to detect the oligomeric form of αS, nor antibodies known to be conformational, like the 5G4 antibody used in Unterberger's studies.

Unlike the Western blot method previously used to detect αS^D in brain homogenates from M83 mice[4], the ELISA did not require a concentration step such as by ultracentrifugation with sarkosyl to detect the disease-associated protein[23]. Disease-associated αS was previously identified in M83 mice by sequential extraction using buffers of increasing strength[2,6]. From a practical point of view, this quantitative assay saves significant time and reagents compared to the Western blot procedure.

In this study, detailed αS molecular analyses using an ELISA approach made it possible to easily quantify immunoreactivity with different antibodies in sick M83 mice. It could shed light on the molecular mechanisms involved in αS aggregation during synucleinopathies from a quantitative viewpoint. This ELISA approach could therefore provide the basis for a fast and easy tool to detect αS^D in various biological samples with a reliable marker of the pathology such as Ser129 phosphorylated αS which Foulds[11] believes shows more promise as a diagnostic marker than the non-phosphorylated protein. Recently, he pointed out the fact that measures of total αS or possibly of non-phosphorylated αS could be used as a surrogate marker for disease progression in PD, as the level of total αS tends to increase over time after the appearance of initial symptoms[24].

Disclosures

The authors have no competing interests to disclose.

Acknowledgements

The authors would like to thank Damien Gaillard for inoculations and follow-up of animal experiments. This work was supported by ANSES (French Agency for Food, Environmental and Occupational Health & Safety) and by a grant from the Foundation France Parkinson.

References

1. Goedert, M., Spillantini, M. G., Del Tredici, K., Braak, H. 100 years of Lewy pathology. *Nat Rev Neurol.* **9**, 13-24 (2013).
2. Waxman, E. A., Giasson, B. I. Specificity and regulation of casein kinase-mediated phosphorylation of alpha-synuclein. *J Neuropathol Exp Neurol.* **67**, 402-416 (2008).
3. Anderson, J. P., *et al.* Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J Biol Chem.* **281**, 29739-29752 (2006).
4. Mougenot, A. L., *et al.* Prion-like acceleration of a synucleinopathy in a transgenic mouse model. *Neurobiol Aging.* **33**, 2225-2228 (2012).
5. Luk, K. C., *et al.* Intracerebral inoculation of pathological alpha-synuclein initiates a rapidly progressive neurodegenerative alpha-synucleinopathy in mice. *J Exp Med.* **209**, 975-986 (2012).
6. Giasson, B. I., *et al.* Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron.* **34**, 521-533 (2002).
7. Luk, K. C., *et al.* Pathological alpha-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science.* **338**, 949-953 (2012).

8. Masuda-Suzukake, M., *et al.* Prion-like spreading of pathological alpha-synuclein in brain. *Brain : a journal of neurology.* **136**, 1128-1138 (2013).
9. Sacino, A. N., *et al.* Amyloidogenic alpha-synuclein seeds do not invariably induce rapid, widespread pathology in mice. *Acta neuropathologica.* **127**, 645-665 (2014).
10. Recasens, A., *et al.* Lewy body extracts from parkinson's disease brains trigger alpha-synuclein pathology and neurodegeneration in mice and monkeys. *Ann Neurol.* (2013).
11. Foulds, P. G., *et al.* Phosphorylated alpha-synuclein can be detected in blood plasma and is potentially a useful biomarker for Parkinson's disease. *FASEB J.* **25**, 4127-4137 (2011).
12. El-Agnaf, O. M., *et al.* Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. *Faseb J.* **20**, 419-425 (2006).
13. Lee, H. J., *et al.* Enzyme-linked immunosorbent assays for alpha-synuclein with species and multimeric state specificities. *J Neruosci Meth.* **199**, 249-257 (2011).
14. Unterberger, U., *et al.* Detection of disease-associated alpha-synuclein in the cerebrospinal fluid: a feasibility study. *Clin Neuropathol.* **33**, 329-334 (2014).
15. Betemps, D., *et al.* Alpha-synuclein spreading in M83 mice brain revealed by detection of pathological alpha-synuclein by enhanced ELISA. *Acta Neuropathol. (Berl).* **2**, 29 (2014).
16. Osman, A. A., *et al.* A monoclonal antibody that recognizes a potential coeliac-toxic repetitive pentapeptide epitope in gliadins). *Eur J Gastroenterol Hepatol.* **13**, 1189-1193 (2001).
17. Pinheiro, J. C., Bates, D. M. Ch. 5. *Mixed-Effects Models in S and S-PLUS.* Chambers, J. **5**, 206-225 Springer (2000).
18. Mougenot, A. L., *et al.* Production of a monoclonal antibody, against human alpha-synuclein, in a subpopulation of C57BL/6J mice, presenting a deletion of the alpha-synuclein locus. *J Neruosci Meth.* **192**, 268-276 (2010).
19. Specht, C. G., Schoepfer, R. Deletion of the alpha-synuclein locus in a subpopulation of C57BL/6J inbred mice. *BMC Neurosci.* **2**, 11 (2001).
20. Lee, B. R., Matsuo, Y., Cashikar, A. G., Kamitani, T. Role of Ser129 phosphorylation of alpha-synuclein in melanoma cells. *J Cell Sci.* **126**, 696-704 (2013).
21. Perrin, R. J., *et al.* Epitope mapping and specificity of the anti-alpha-synuclein monoclonal antibody Syn-1 in mouse brain and cultured cell lines. *Neurosci Lett.* **349**, 133-135 (2003).
22. Emmanouilidou, E., *et al.* Assessment of alpha-synuclein secretion in mouse and human brain parenchyma. *PLoS One.* **6**, e22225 (2011).
23. Mougenot, A. L., *et al.* Transmission of prion strains in a transgenic mouse models overexpressing human A53T mutated alpha-synuclein. *J Neuropathol Exp Neurol.* **70**, 377-385 (2011).
24. Foulds, P. G., *et al.* A longitudinal study on alpha-synuclein in blood plasma as a biomarker for Parkinson's disease. *Sci Rep.* **3**, 2540 (2013).

