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Thierry Baron, Jean-Noël Arsac, Jérémy Verchère, Habiba Tlili, Claire Aufauvre, et al.. Co-expression of APP/PS1 disrupts the distribution of brain lesions in a synucleinopathy transgenic mouse model (M83). *Acta Neuropathologica*, 2022, 143 (4), pp.527-529. 10.1007/s00401-022-02410-x. hal-04057816

**HAL Id: hal-04057816**

**<https://hal.science/hal-04057816v1>**

Submitted on 23 Feb 2024

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**Co-expression of APP/PS1 disrupts the distribution of brain lesions in a  
synucleinopathy transgenic mouse model (M83)**

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**Keywords:** alpha-synuclein, beta-amyloid, Parkinson's, strain, aggregation

1 Human neurodegenerative diseases (HNDs) often involve the aggregation of several proteins,  
2 with disease resulting from their interactions rather than their coincidental co-existence [1, 10].  
3 Dementia with Lewy bodies is characterized by alpha-synuclein (aS) aggregation and most  
4 cases show Alzheimer's disease neuropathology involving amyloid plaques and neurofibrillary  
5 tangles [5], which is associated with earlier onset of dementia. This suggests synergistic  
6 interactions of aS with  $\beta$ -amyloid or/and tau proteins.

7 Here, we performed a pilot study in which we examined aging of a cohort of 13 mice  
8 obtained by breeding M83 homozygous (Ho) mice, expressing human A53T mutated aS [4],  
9 with APPPS1-21 hemizygous (He) mice expressing human mutated amyloid precursor (APP)  
10 and presenilin-1 (PS1) proteins [9]. Mice were sacrificed at the age of 22 months, beyond which  
11 M83 He mice can develop signs of paralysis, occurring in old M83 mice. None of the mice had,  
12 however, developed paralysis, even the 6 APPxM83 mice bearing the APP/PS1 transgene.  
13 Surprisingly, Western blot analyses of samples from the cerebral cortices of the 13 mice showed  
14 abundant aggregated serine 129 phosphorylated aS (PSer129-aS) in 3 of the 6 APPxM83 mice,  
15 instead of only monomeric aS in all the other mice (Fig. 1a). We further examined PSer129-aS  
16 neuroanatomical distribution using both Western blot and ELISA, which showed the highest  
17 levels in the cerebral cortex, hippocampus and striatum (Fig. 1b and Supplementary Fig.1),  
18 where lesions are scarce or absent in sick M83 mice [4]. PSer129-aS was found at lower levels  
19 in the midbrain and brainstem, where it is consistently more abundant in sick M83 mice. This  
20 striking difference was confirmed by immunohistochemistry, as illustrated by major and  
21 unexpected somatodendritic and neuritic PSer129-aS labelling in the hippocampus and cerebral  
22 cortex of APPxM83 mice (Fig. 1c-e and Supplementary Fig. 2c), where it is typically absent or  
23 scarce in sick M83 mice. Remarkably, the amyloid plaque load was strongly reduced in  
24 APPxM83 mice compared to age-matched APPPS1-21 mice (Supplementary Fig. 2a-b). The  
25 direct molecular interactions of aS with APP could favor the accumulation of phosphorylated

1 aggregation-prone aS conformers in the brain regions with the highest levels of both aS and  
2 APP expression (Supplementary Fig. 3)[10]. An effect of mutated PS1 on aS phosphorylation  
3 has also been reported in humans [6].

4         Additionally, intra-striatal inoculation in M83 (Ho) of a cerebral cortex homogenate  
5 from one of the APPxM83 mice containing P<sub>Ser129</sub>-aS triggered accelerated paralysis of M83  
6 (Ho) mice, which was not the case after inoculations of two P<sub>Ser129</sub>-aS negative cerebral  
7 cortex homogenates from APPxM83 or M83 (He) mice (Supplementary Fig. 4). Typical  
8 distribution of P<sub>Ser129</sub>-aS in the hindbrain was observed in these paralyzed M83 (Ho) mice.  
9 Recent studies suggested that aS “strains” may explain the phenotypic diversity of  
10 synucleinopathies [3, 7, 8, 11]. One of these studies suggested that “strains” could be  
11 differentiated in M83 mice inoculated with distinct sources of aggregated aS, based on the  
12 finding of more abundant P<sub>Ser129</sub>-aS deposits in the hippocampus and cerebral cortex [7].  
13 Another study showed increased pathogenicity and lesions in M83 mice challenged by  
14 recombinant aS, when its aggregation was initially triggered in the presence of p25 $\alpha$  protein  
15 involved in the lesions of multiple system atrophy in humans [3].

16         Distinct conformations of these proteins, similar to prion “strains”, may help to explain  
17 the phenotypic diversity of HNDs. However, another, not mutually exclusive, hypothesis  
18 related to the multiple pathologies within the multi-morbid old brain, has been poorly  
19 considered [2]. Altogether, our data reinforce the idea that interactions of aS with other cerebral  
20 components may play a key role in determining its pathogenic behavior.

21

22 **Fig. 1** Co-expression of APP/PS1 leads to distinct distribution of brain lesions in M83 mice.

23 **(a)** Western blot detection of P<sub>Ser129</sub>-aS in brain homogenates from cerebral cortices of M83  
24 (He) mice (Nos. 1 to 7) or APPxM83 mice (Nos. 1 to 6). High molecular weight (HMW) aS  
25 species are seen (lower panel) in three of the six mice expressing APP (6E10 antibody) (upper

1 panel). **(b)** Western blot and ELISA detection of P<sub>Ser129</sub>-a $\beta$ S in the brain of an APPxM83  
2 mouse (No. 4) compared to a 19-month-old sick M83 mouse. ELISA threshold (dashed line)  
3 represents means + 3xSD of three repeated ELISA tests on two half-brains from 5-month-old  
4 M83 mice. OB: olfactory bulbs, CTX: cerebral cortex, STR: striatum, HIP: hippocampus, MB:  
5 midbrain, CB: cerebellum, BS: brainstem. **(c)** Immunohistochemical 6E10 staining in the  
6 hippocampus of an M83 (He) mouse (No. 3) or APPxM83 mouse (No. 1). Scale bar: 500  $\mu$ m.  
7 **(d, e)** p<sub>Ser129</sub>-a $\beta$ S staining in hippocampal regions CA1 (d) and dentate gyrus (e) of an M83  
8 (He) mouse (No. 7) or APPxM83 mouse (No. 3). Scale bar: 50  $\mu$ m.

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## 19 **Acknowledgements**

20 We gratefully acknowledge Mathias Jucker for providing the APPPS1-21 mice and for helpful  
21 discussions, as well as Damien Gaillard, Muris Humo and Latifa Lakhdar for the follow-up of  
22 animal experiments.

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## 24 **Declarations**

## 25 **Conflict of interest**

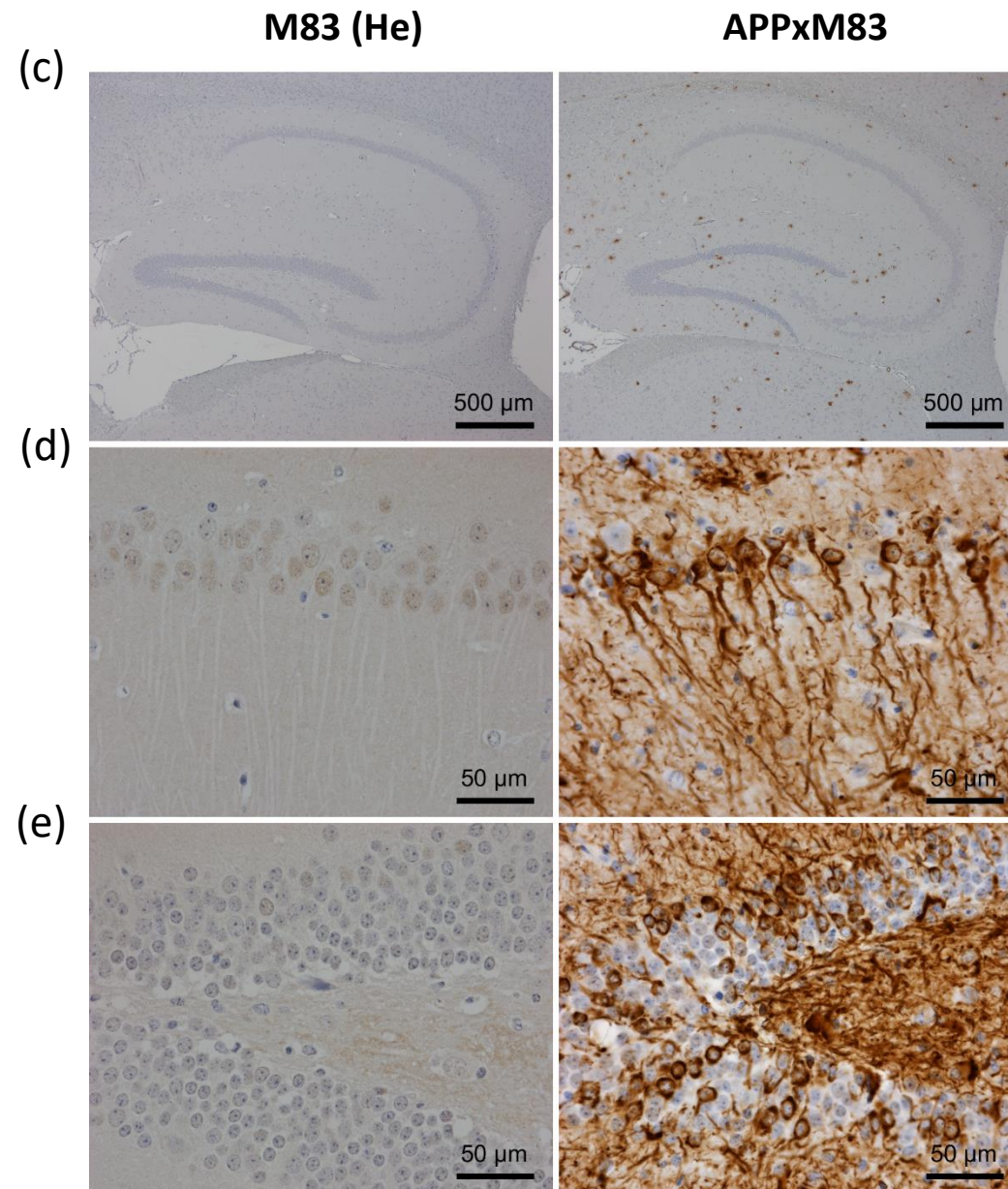
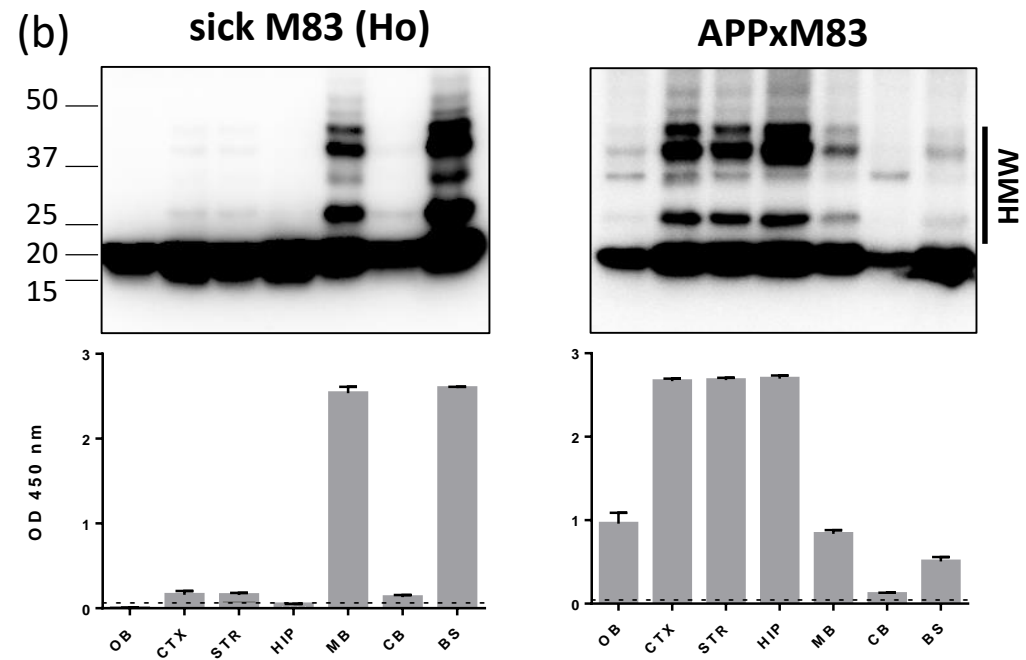
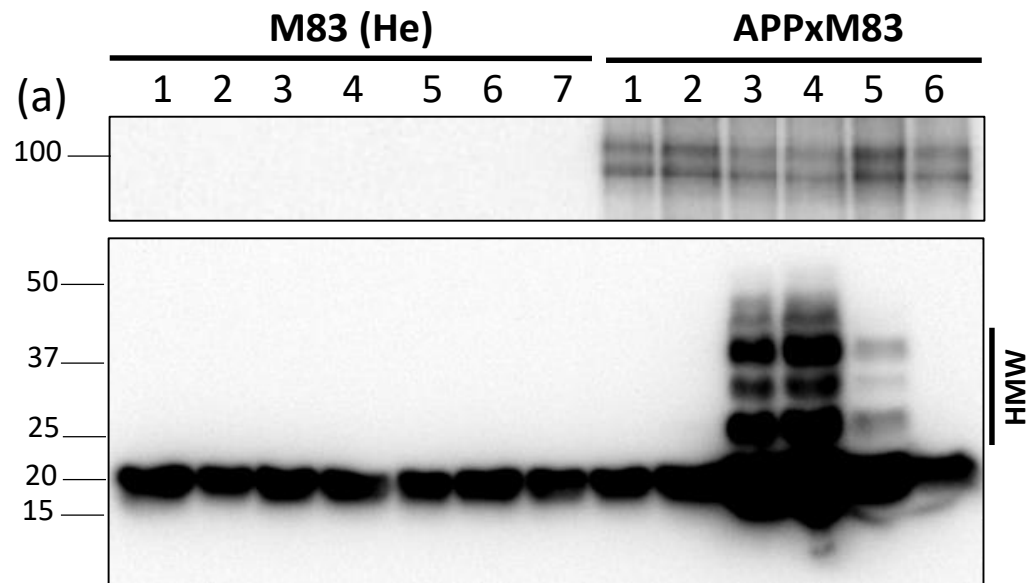
26 The authors declare no actual or potential conflict of interest.

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## 28 **Ethics approval**

1 The experimental studies were authorized by Ethics Committee CE2A - 16, ComEth  
2 ANSES/ENVA/UPEC and by the French Ministry of Higher Education, Research and  
3 Innovation (refs 19-019 and 19-042).

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## **Supplementary Information**

### **Co-expression of APP/PS1 disrupts the distribution of brain lesions in a synucleinopathy transgenic mouse model (M83)**

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## Materials and Methods

### Animals

M83 transgenic mice (B6;C3H-Tg[SNCA]83Vle/J) express A53T mutated human alpha-synuclein (aS) protein under the control of the prion protein promoter [2]. Homozygous (Ho) M83 mice 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 [2]. These clinical signs may only occur later, beyond the age of 22 months, in hemizygous (He) mice. At the terminal stage, brain lesions, including intraneuronal deposits of serine 129 phosphorylated aS (PSer129-aS), are consistently prominent in the midbrain, brainstem and deep cerebellar nuclei, whereas they remain scarce or absent in most cortical brain regions or in the hippocampus, including after intracerebral inoculations of aggregated aS [1-3, 5, 6].

APPPS1-21 transgenic mice (B6.Cg-Tg(Thy1-APP<sup>Sw</sup>,Thy1-PSEN1\*<sup>L166P</sup>)21Jckr) contain human transgenes for both amyloid precursor protein (APP), bearing the Swedish mutation, and presenilin 1 (PS1), containing an L166P mutation, both under the control of the Thy1 promoter [4]. Amyloid deposits appear at the age of 6 weeks in the neocortex then spread in the hippocampus and widely throughout most of the brain regions.

Homozygous M83 mice and hemizygous APPPS1-21 mice were bred to produce a progeny from which 13 animals were followed up to the age of 22 months. Genotypes were determined with selected forward (GAATTCGACATGACTCAGG) and reverse (GTTCTGCTGCATCTTGGACA) primers in order to identify hemizygous M83 mice presenting or not the APP/PS1 genes. The APP expression was further checked by Western blot analysis with the 6E10 antibody (1:2000; Biolegend, 803001, San Diego, USA).

The animals 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 (No. D69387 0801) for breeding and experimental studies, in accordance with EEC Directive 86/609/EEC and French decree No. 2013-118. The experimental studies were authorized by the Ethics Committee CE2A - 16, ComEth ANSES/ENVA/UPEC and by the French Ministry of Higher Education, Research and Innovation (refs 19-019 and 19-042).

### **Biochemical detection of alpha-synuclein**

Biochemical analyses included analyses of P<sup>Ser129</sup>-a $\beta$  accumulation and of human/murine a $\beta$  expression in 22-month-old or 5-month-old APP $\times$ M83 and M83 (He) mice.

a $\beta$  extractions from dissected brain homogenates was obtained in High Salt (HS) Buffer (50 mM Tris-HCl pH 7,5, 750 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% phosphatase and protease inhibitor cocktail).

For Western blot analyses brain homogenates were mixed with TD4215 denaturing buffer (final concentration: 4% SDS, 2%  $\beta$  mercaptoethanol, 192 mM glycine, 25 mM Tris, 5% sucrose) in order to load 20 or 2  $\mu$ g proteins per lane for P<sup>Ser129</sup>-a $\beta$  or human/murine a $\beta$  respectively, after assessment of proteins concentrations with DC protein Assay (Biorad, Marnes-La-Coquette, France). Proteins were separated after heat denaturation (5 min at 100°C) on 12% TGX Stain Free FastCast acrylamide gels or TGX FastCast acrylamide gels (Biorad, Marnes-La-Coquette, France) loaded with denatured proteins, then blotted onto PVDF membranes (Immobilon-P, Sigma, Saint-Quentin Fallavier, France). After migration, Stain Free gels were activated for 1 min on Chemidoc MP apparatus (BioRad, Marnes-La-Coquette, France) and image analysis was done using ImageLab software (BioRad) to control the protein loads per lane. a $\beta$  was cross-linked to the membranes using 4% paraformaldehyde and 0.01% glutaraldehyde diluted in PBS for 30 minutes and unspecific binding sites were saturated by adding 5% milk for 1 h. a $\beta$  was detected by the rabbit monoclonal EP1536Y antibody (1:1000;

Abcam, Paris, France) for P<sub>Ser129</sub>-a<sub>S</sub>, or rabbit monoclonal antibodies MJFR1 (1:4000; Abcam, Paris, France) or D37A6 (1:1000; Ozyme, Saint-Cyr-l'Ecole, France), for human or murine a<sub>S</sub> respectively. GAPDH was detected by the mouse monoclonal antibody 6C5 (1:4000; Sigma, Saint-Quentin Fallavier, France). The antibodies were incubated overnight at +4°C. After washings, membranes were then incubated with anti-mouse or anti-rabbit HRP (1:10000; Clinisciences, Nanterre, France), for 1 h at room temperature (RT). HRP was then visualized with chemiluminescent substrate (Supersignal WestDura, 10445345, Fisher, Illkirch, France) and analyzed using the ChemiDoc system and ImageLab software (Bio-Rad).

For ELISA analyses, P<sub>Ser129</sub>-a<sub>S</sub> was detected from mouse brain homogenates as previously described [5]. Briefly, plates (MaxiSorp<sup>TM</sup>, Fisher, Illkirch, France) were coated with Syn303 (1:1000; Biolegend, San Diego, USA) antibody in 50mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (pH9.6) with 100 µl per well at 4°C overnight. Plates were washed 5 times in PBS with 0.05% Tween 20 (PBST). Superblock T20 PBS blocking buffer (37536, Fisher, Illkirch, France) was then added for 1h at room temperature (RT) with shaking at 150 rpm. The plates were again washed 5 times in PBST and, after protein dosage by DC Protein Assay (Biorad, Marnes-La-Coquette, France), 20 µg proteins in PBST BSA 1% were incubated at 25°C for 2h with shaking. After washing 5 times with PBST, captured a<sub>S</sub> was detected with EP1536Y antibody (1:3000; Abcam, Paris, France) in PBST 1% BSA; plates were incubated for 1 h at 25°C under agitation. The plates were washed another 5 times with PBST, and anti-rabbit IgG HRP conjugate (1: 2000; 4010-05, Clinisciences, Nanterre, France) was added in PBST-1% BSA; plates were incubated for 1 h at 25°C. After washing the plates 5 times with PBST, 100 µL of 3,3',5,5'-tetramethylbenzidine solution (T0440, Sigma, Saint-Quentin Fallavier, France) were added to each well and plates were incubated for 15 min with shaking. The reaction was stopped with 100 µL of 1 N H<sub>2</sub>SO<sub>4</sub>, and the absorbance was measured at 450 nm with the Clariostar (BMG) microplate reader. The threshold was calculated as the mean + 3xSD of three repeated

ELISA tests on two half-brains from 5-month-old M83 mice. For detection of human and murine aS, the following modifications of the above ELISA protocol were performed. Plates were coated with clone 42 antibody (1:2500; BD Biosciences, Pont de Claix, France) in 50mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (pH9.6) at 4°C overnight. Captured aS (0,5 µg of protein for human aS and 2 µg of protein for murine aS) was detected either by rabbit monoclonal antibodies MJFR1 (1:25000; Abcam, Paris, France) or D37A6 (1:1000; Ozyme, Saint-Cyr-l'Ecole, France) for human or murine aS respectively. The threshold was calculated as the mean + 3xSD of three repeated ELISA on half-brains from C57Bl/6 mice devoid of aS.

### **Immunohistochemical detection of P<sub>Ser129</sub>-aS deposits and amyloid plaques**

Brain samples were fixed in 4% paraformaldehyde and paraffin embedded to be cut into serial 5 µm coronal brain sections. Slices were deparaffinized and rehydrated. For the detection of P<sub>Ser129</sub>-aS, brain sections were treated with a citrate solution (C9999, Sigma, Saint-Quentin Fallavier, France) with heat antigen retrieval. Once refrigerated, the brain sections were treated by a 4 M solution of guanidinium thiocyanate (EU0046, Euromedex, Souffelweyersheim, France) during 20 min. For the detection of amyloid plaques we did a pretreatment with 90% formic acid at room temperature (RT) for 20 min, followed by washing in an excess of water. The endogenous peroxidase activity was blocked by a 5 min immersion in a 3% hydrogen peroxide solution. The non-specific binding was blocked by incubating the sections in a solution of Blocking Reagent (11096176001, Roche, Boulogne-Billancourt, France) for 30 min. Subsequently, the sections were incubated overnight at 4°C with primary antibodies rabbit anti-P<sub>Ser129</sub>-aS EP1536Y (1:300; Abcam, Paris, France) or mouse anti-β-amyloid 6E10 (1:200; Biologend, San Diego, USA ) antibodies. The histological sections were blocked a second time by incubating the sections in the Solution Blocking Reagent (11096176001, Merck, Saint-Quentin Fallavier, France) for 30 min. The HRP-labeled anti-rabbit Ig secondary antibody (1:250; 4010-05, Cliniscience, Nanterre, France) or anti-mouse Ig secondary antibody (1:500;

1010-05, Cliniscience, Nanterre, France) were incubated during 1.5 h at room temperature. HRP enzymatic reaction was revealed using ImmPACT DAB ® peroxidase (HRP) substrate (SK-4105, Eurobio Scientific, Les Ulis, France) for 8 min at room temperature then washed in water. The slides were then counterstained with aqueous hematoxylin. The stained sections were observed under a light microscope BX51 (Olympus) coupled to INFINITY3-6UR camera (Lumenera) and lumenera software (Infinity analyze version 6.5.5) was used.

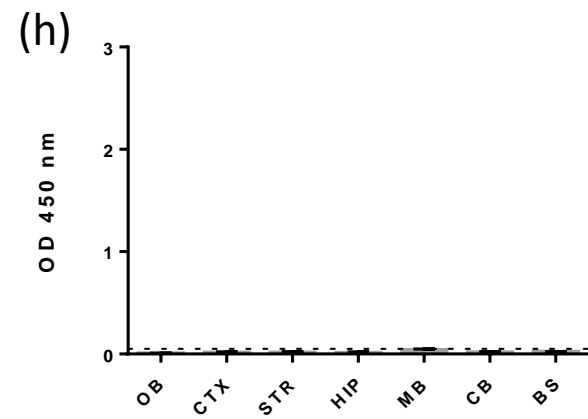
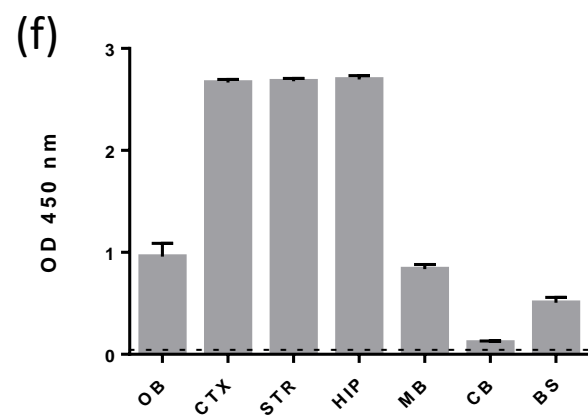
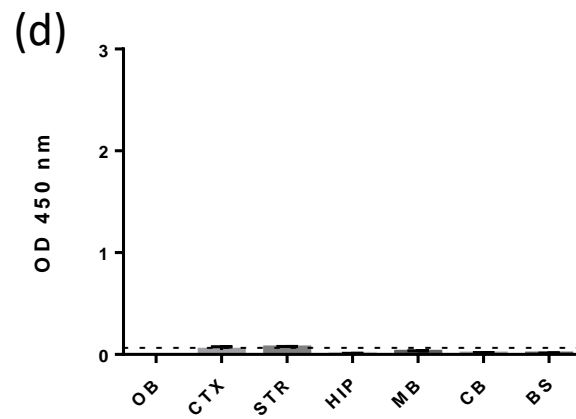
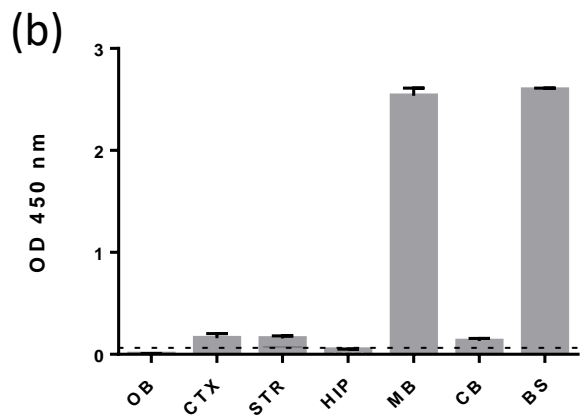
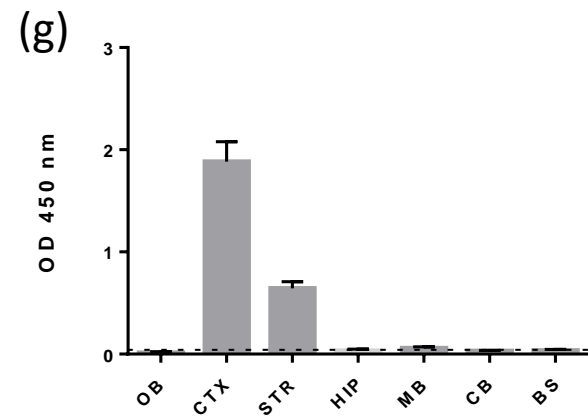
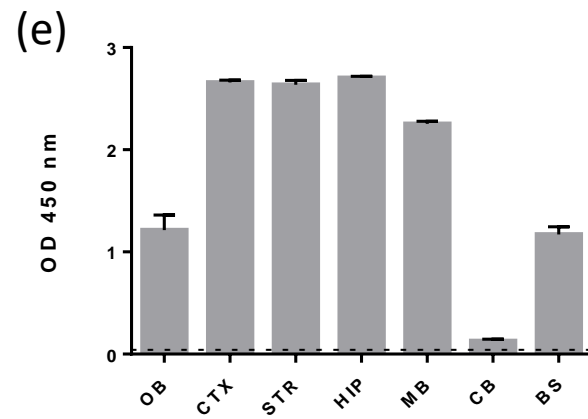
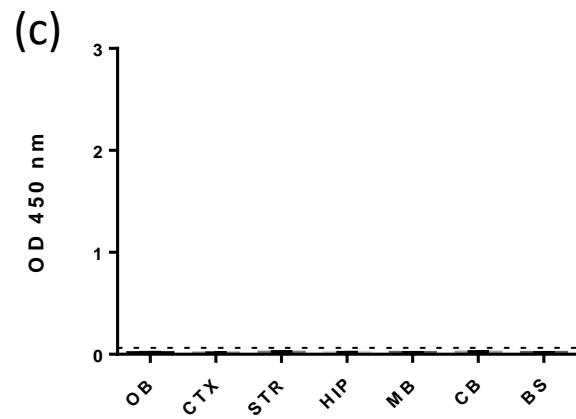
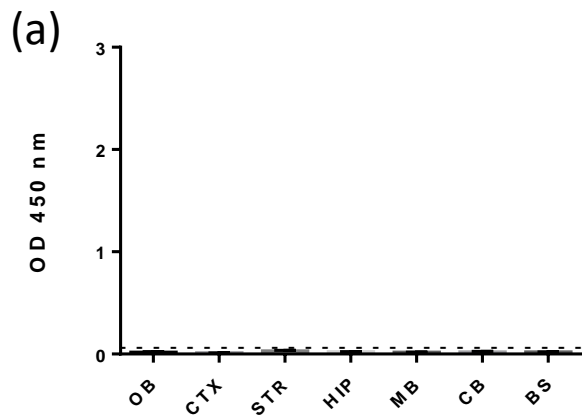
### **Bioassays**

To examine the seeding capacity of the cerebral cortex of APPxM83 mice according to the presence or not of Ser129P-aS, homogenates (2% (wt/vol)) were prepared from the cerebral cortices of two 22-month-old APPxM83 mice containing or not P<sub>Ser129</sub>-aS (APPxM83 mice Nos. 3 and 6 respectively) and from one of the 22-month-old M83 hemizygous mice (M83 No. 7). 2 µl (40 µg brain equivalent) were stereotactically injected into the left striatum (anteroposterior (AP): +0.014, mediolateral (ML): +0.2, dorsoventral (DV): - 0.275) of 2 month old homozygous M83 mice. An additional control experiment was performed by intra-striatal injection of a midbrain homogenate from a 20-month-old sick M83 mouse, as previously described [1]. Mice were observed daily and were sacrificed when they developed the first symptoms of the typical M83 disease, including slow ambulation, balance disorders and partial paralysis of a hind limb. The M83 disease was confirmed by P<sub>Ser129</sub>-aS ELISA detection as previous described [5], in a sample of the cervical spinal cord.

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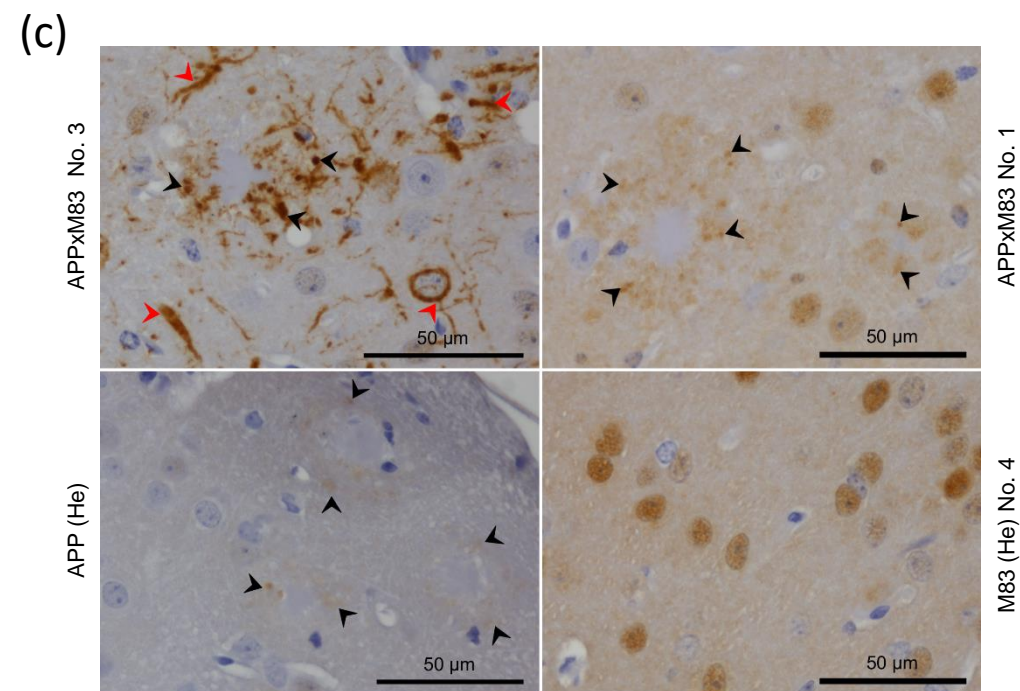
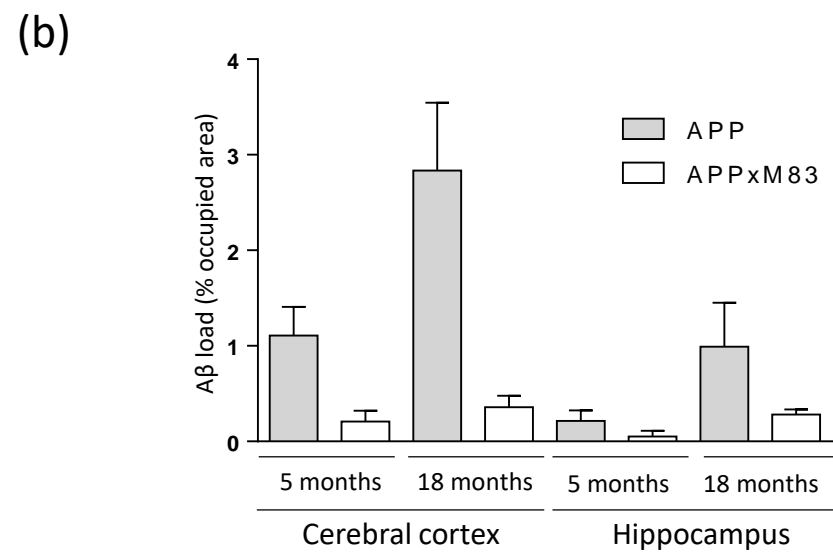
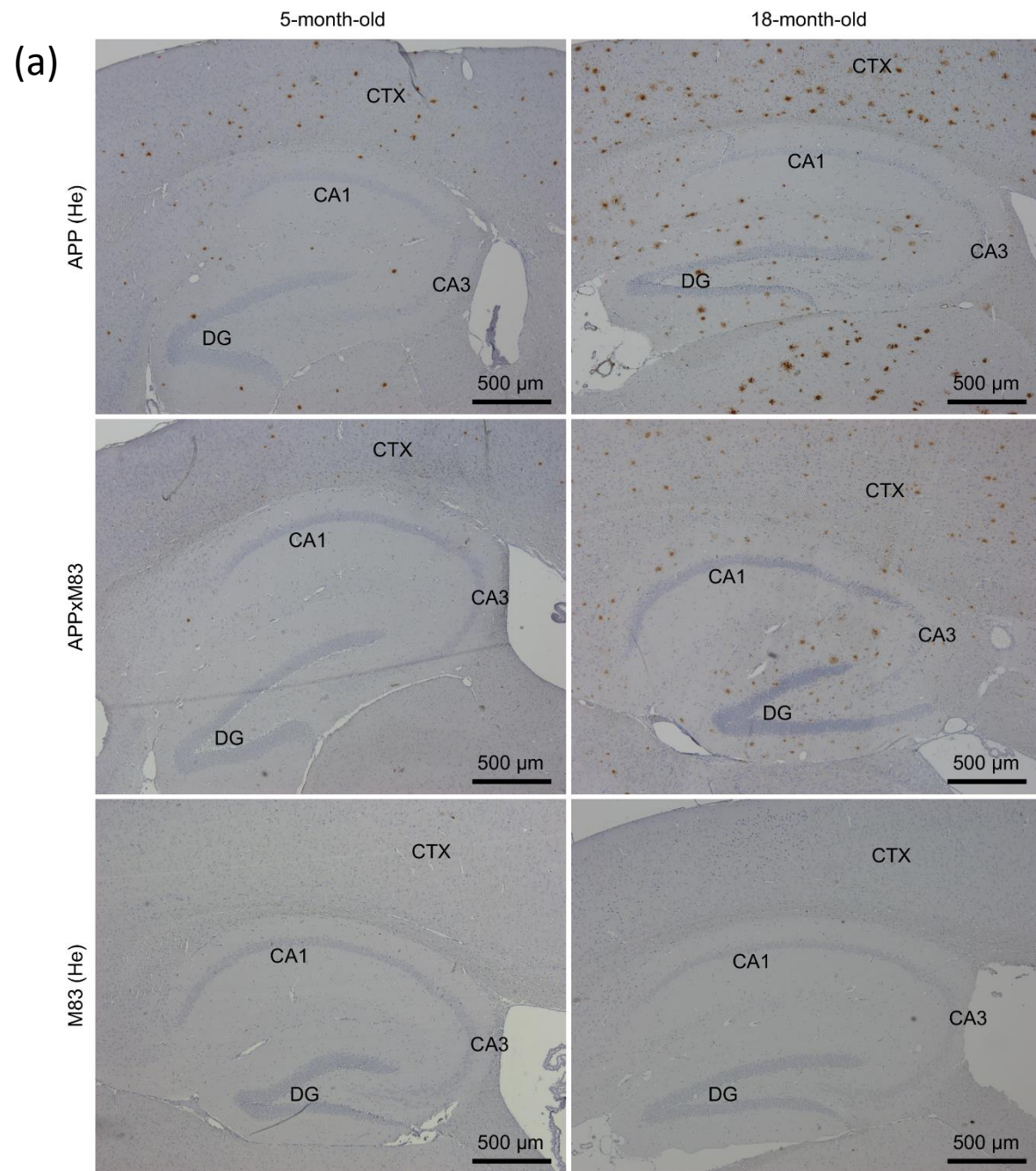
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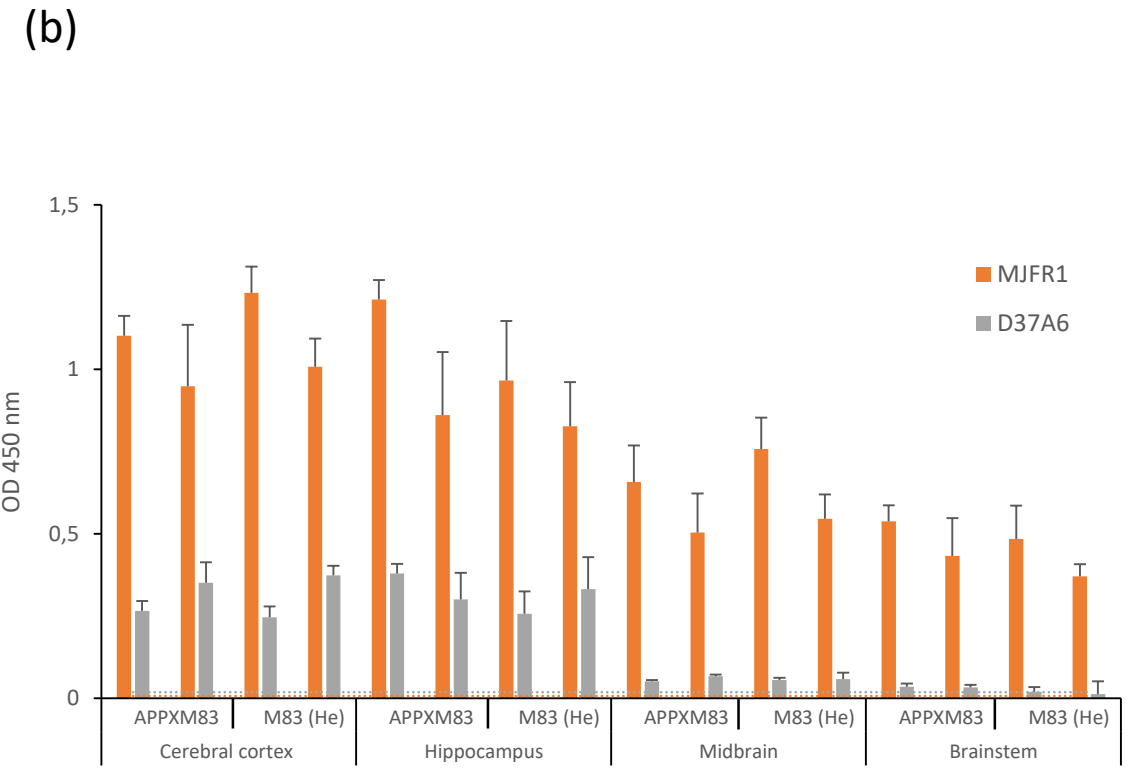
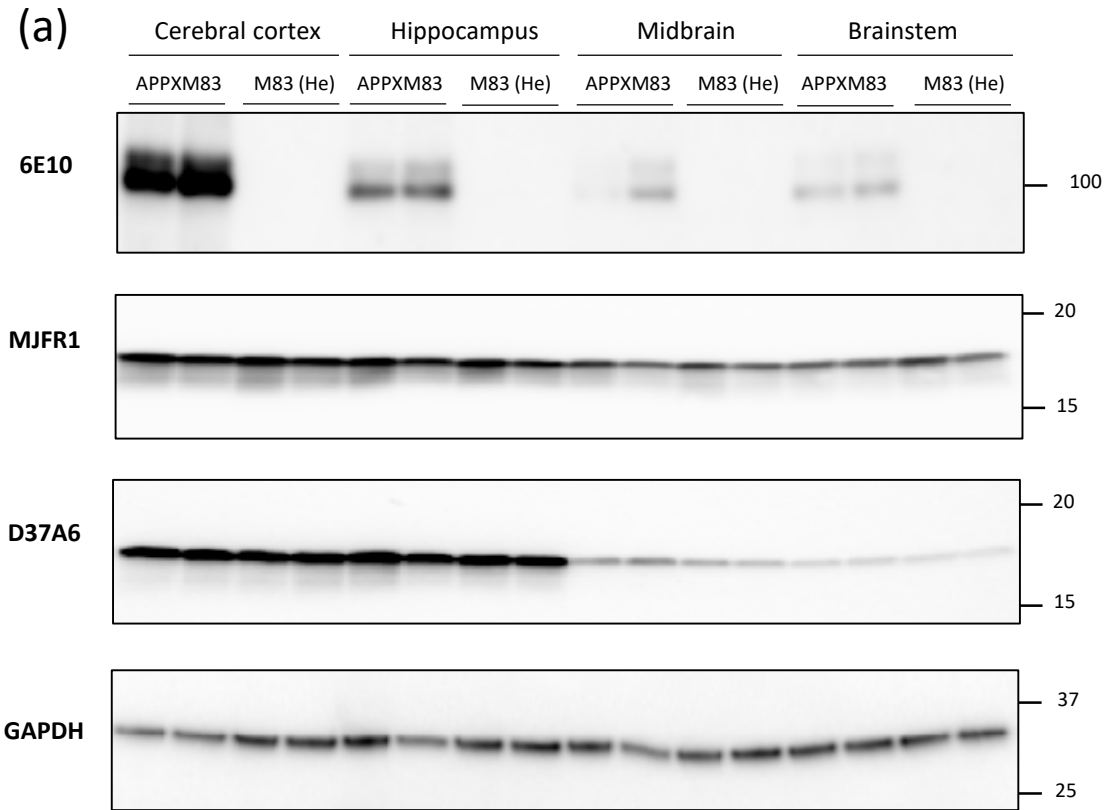




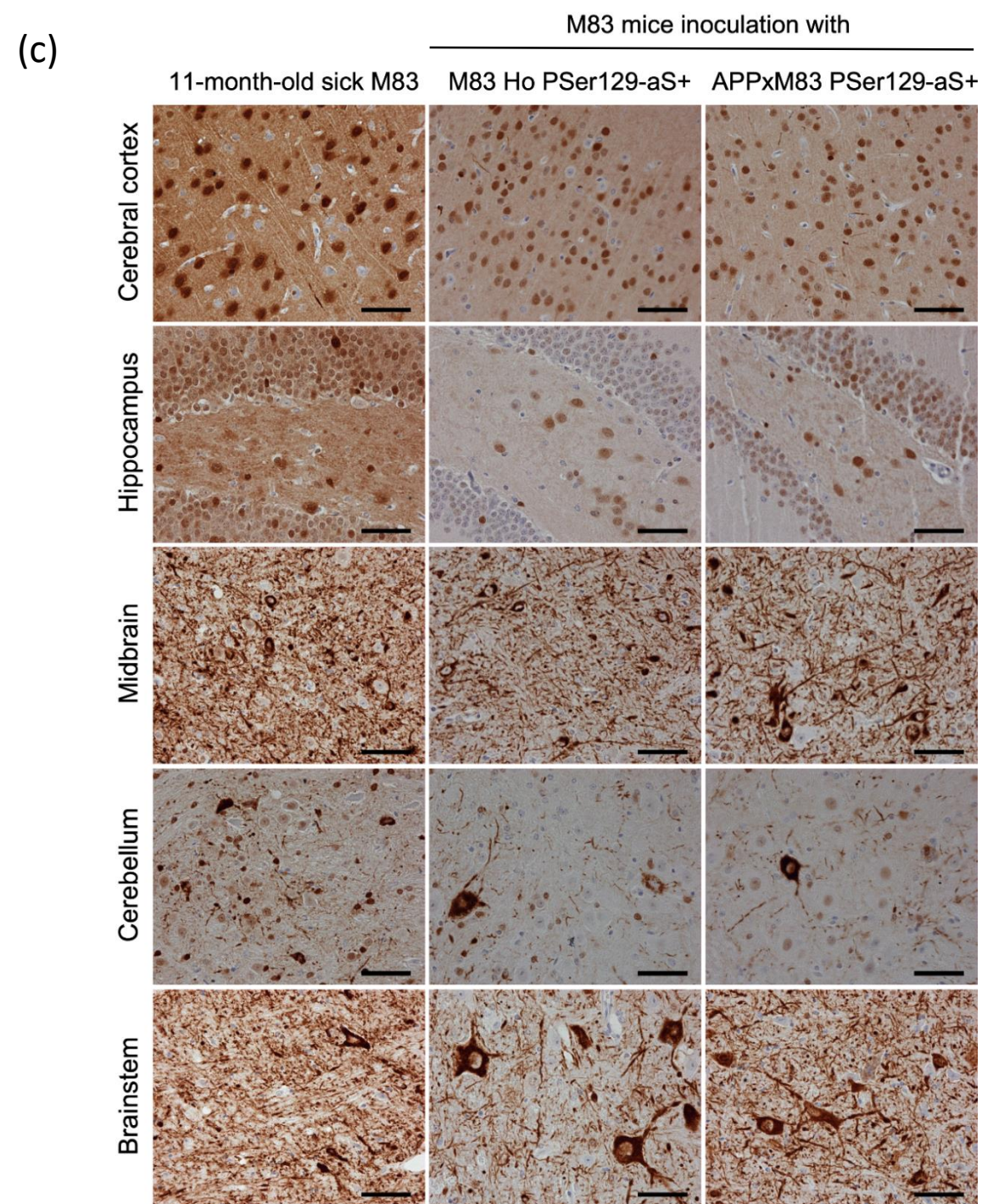
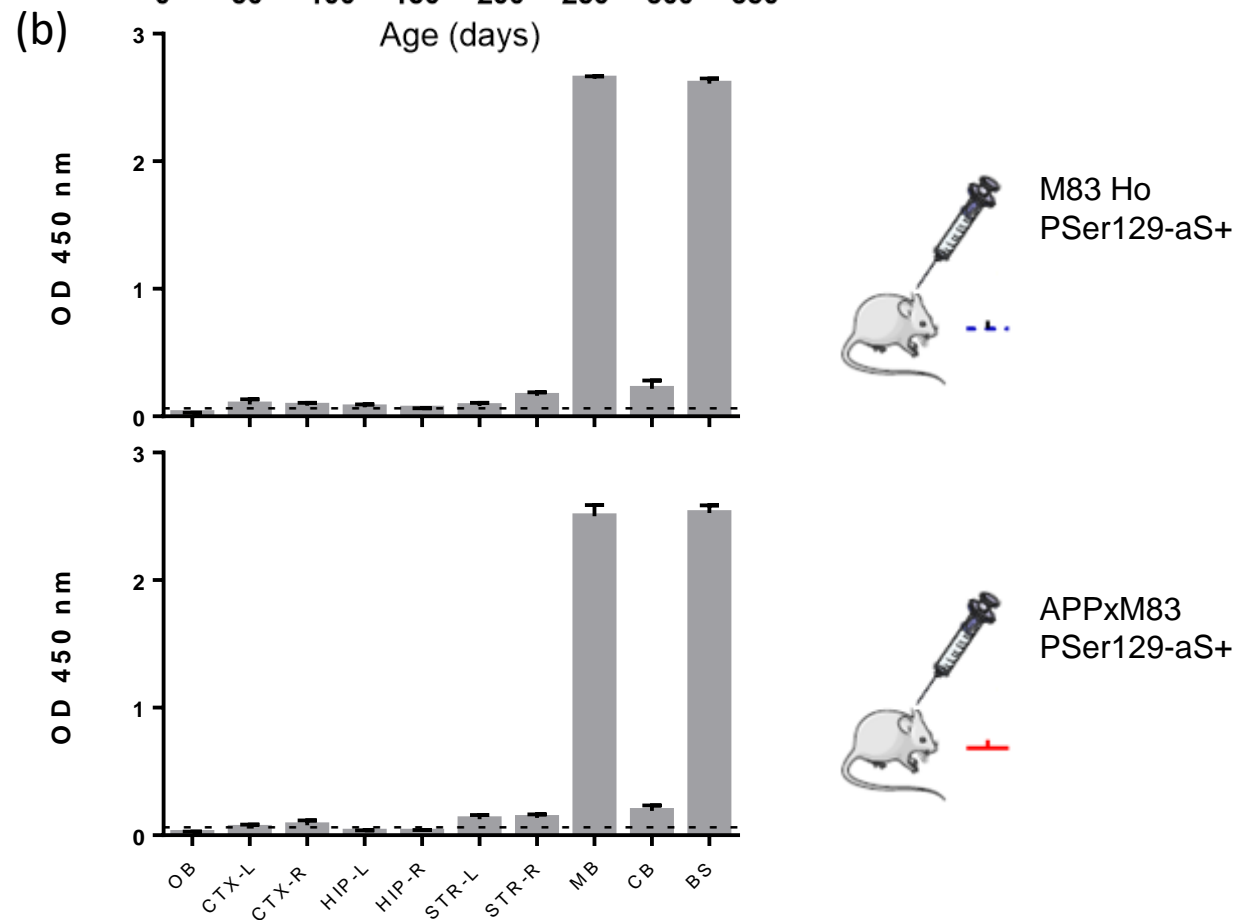
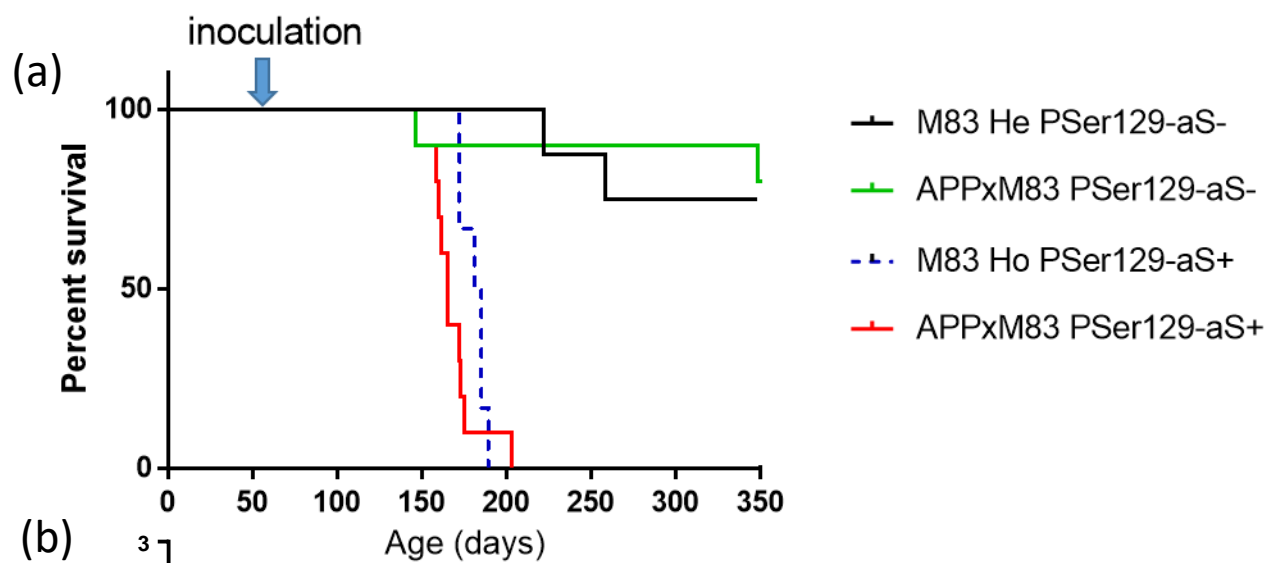
**Figure S1.** Detection of serine 129 phosphorylated alpha-synuclein (PSer129-aS) in APPxM83 mice. Brain homogenates of 7 brain regions were analyzed by ELISA using Syn303-EP1536Y antibodies as previously described [5], from one of the 7 M83 hemizygous (He) mice (No. 7) (a), a 19-month-old sick M83 homozygous (Ho) mouse (b) and the 6 APPxM83 mice (Nos. 1 to 6 respectively in c-h). The dashed line indicates a cut-off level determined as means + 3xSD measured in samples from half-brains from two 5-month-old M83 mice. The results were obtained from three repeated measures of immunoreactivity (OD 450 nm). OB: olfactory bulbs, CTX: cerebral cortex, STR: striatum, HIP: hippocampus, MB: midbrain, CB: cerebellum, BS: brainstem.



**Figure S2.** Co-expression of human A53T mutated aS in APPxM83 mice reduces  $\beta$ -amyloid load in the cerebral cortex and hippocampus and promotes widespread deposits of serine 129 phosphorylated aS. (a) Representative images of amyloid plaques (6E10) staining in the APP (He), APPxM83 or M83 (He) mouse brain at the ages of 5 or 18 months, showing reduced amyloid plaques in the cerebral cortex and hippocampus of APPxM83 mice. Abbreviations: CA1 region of the hippocampus (CA1); CA3 region of the hippocampus (CA3); dentate gyrus (DG); cerebral cortex (CTX). Scale bar = 500  $\mu$ m. (b) Quantification was performed by measuring the percentage-occupied area of the 6E10 labelled surface in the cerebral cortex and hippocampus (n = 3 for APP (He) or n=2 for APPxM83 mice). (c) Representative images of P<sub>Ser129</sub>-aS labelling in the cerebral cortex of 22-month-old APPxM83 (Nos. 1 and 3) and M83 (He) (No. 4) mice or of a 18-month-old APP (He) mouse. Note that in the APP (He) mouse P<sub>Ser129</sub>-aS staining is present, located around amyloid plaques (black arrowheads), which is increased in APPxM83 mice. In an APPxM83 mouse with the highest levels of P<sub>Ser129</sub>-aS (No. 3), abundant P<sub>Ser129</sub>-aS deposits are also present as widespread intracytoplasmic and neuritic deposits (red arrowheads), independently of amyloid plaques. Scale bar = 50  $\mu$ m.



**Figure S3.** Both human and murine aS are similarly overexpressed in rostral brain regions in both M83 and APPxM83 mice. (a) Western blot detection of human aS (MJFR1 antibody) and murine aS (D37A6 antibody) in brain homogenates from the cerebral cortices, hippocampus, midbrain and brainstem of 5-month-old APPxM83 or M83 (He) mice. In the upper panel, amyloid precursor protein (APP) was detected by 6E10 antibody specifically in APPxM83 mice, showing higher APP levels in the cerebral cortex and hippocampus. Higher levels of both human and murine aS were also detected in these rostral brain regions in both APPxM83 and M83 (He) mice. Comparable amounts of brain materials were loaded in each lane, as shown by an anti-GAPDH antibody used as a loading control. Bars to the right of the Western blot indicate the molecular weight markers (kDa). (b) Detection of human or murine aS in brain homogenates from the cerebral cortices, hippocampus, midbrain and brainstem of 5-month-old APPxM83 or M83 (He) mice. Higher levels of both human and murine aS were similarly detected in the rostral brain regions. The dashed line indicates a cut-off level determined as means + 3xSD measured in samples from half-brains from C57Bl/6 mice devoid of aS. The results were obtained from 4 repeated measures of immunoreactivity (OD 450 nm).



**Figure S4.** Typical phenotypic features of M83 mice are found after intra-striatal inoculation of a sample of cerebral cortex of APPxM83 mouse containing PSer129-aS. (a) Survival of M83 (Ho) mice after intra-striatal inoculation of homogenates from cerebral cortices of APPxM83 mice containing (No. 3) (red line) or not (No. 6) (green line) PSer129-aS or of a M83 He mouse (No. 7) (PSer129-aS negative) (black line). An additional experiment (blue line) illustrates a control by intra-striatal inoculation of a midbrain homogenate from a 20-month-old sick M83 mouse. All the mice inoculated with APPxM83 mouse No. 3 showed the typical onset of paralysis, and were positive in the cervical spinal cord by ELISA with Syn303-EP1536Y antibodies. (b) Distribution of PSer129-aS immunoreactivities in the different brain regions of M83 mice after intra-striatal inoculations of the brain homogenates from the 20-month-old sick M83 mouse (upper panel) or the APPxM83 mouse (No. 3) (lower panel) containing PSer129-aS. In both cases PSer129-aS accumulates mainly into the midbrain and brainstem. (c) Immunohistochemical staining of PSer129-aS in 5 brain regions of an 11-month-old sick M83 mouse (left column) and of sick M83 mice inoculated into the striatum with the midbrain homogenate from the 20-month-old sick M83 mouse (middle column) or the cerebral cortex homogenate from the APPxM83 mouse (No. 3) (right column) containing PSer129-aS. In the three mice, somatodendritic and neuritic deposits are mainly identified in the midbrain and brainstem, and in the deep cerebellar nuclei, whereas such deposits are scarce or absent in the cerebral cortex and hippocampus. Scale bar: 50  $\mu$ m.