

# Prion infection promotes extensive accumulation of $\alpha$ -synuclein in aged human $\alpha$ -synuclein transgenic mice

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In neurodegenerative disorders of the aging population, misfolded proteins, such as PrP<sup>Sc</sup>,  $\alpha$ -synuclein, amyloid  $\beta$  protein and tau, can interact and result in enhanced aggregation, cross seeding and accelerated disease progression. Previous reports have shown that in Creutzfeldt-Jakob disease and scrapie,  $\alpha$ -synuclein accumulates near PrP<sup>Sc</sup> deposits. However, it is unclear if pre-existing human  $\alpha$ -synuclein aggregates modified prion disease pathogenesis, or if PrP<sup>Sc</sup> exacerbates the  $\alpha$ -synuclein pathology. Here, we inoculated infectious prions into aged  $\alpha$ -synuclein transgenic (tg) and non-transgenic littermate control mice by the intracerebral route. Remarkably, inoculation of RML and mNS prion strains into  $\alpha$ -synuclein tg mice resulted in more extensive and abundant intraneuronal and synaptic  $\alpha$ -synuclein accumulation. In addition, infectious prions led to the formation of perineuronal  $\alpha$ -synuclein deposits with a neuritic plaque-like appearance. Prion pathology was unmodified by the presence of  $\alpha$ -synuclein. However, with the mNS prion strain there was a modest but significant acceleration in the time to terminal prion disease in mice having  $\alpha$ -synuclein aggregates as compared to non-tg mice. Taken together, these studies support the notion that PrP<sup>Sc</sup> directly or indirectly promotes  $\alpha$ -synuclein pathology.

DO NOT DISSEMINATE.

## Introduction

Misfolded protein aggregates are pathologic hallmarks for many neurodegenerative disorders, including Alzheimer, Parkinson and prion disease.<sup>1</sup> Protein aggregates are typically  $\beta$ -sheet rich oligomers or fibrils that occur as intracellular or extracellular deposits in the central nervous system.<sup>2</sup> In patients with neurodegenerative disease, two or more protein aggregates commonly co-exist, for example  $\alpha$ -synuclein and amyloid  $\beta$  in dementia with Lewy bodies<sup>3,4</sup> and tau and amyloid  $\beta$  in Alzheimer disease.<sup>5</sup> Consistent with these observations, a patient was recently reported that had suffered from a rapidly progressive dementia with multiple aggregated proteins existing in the brain, including prions, tau,  $\alpha$ -synuclein and amyloid  $\beta$ ,<sup>6</sup> which may suggest a general disturbance in protein folding or a protein aggregate catalyzing the aggregation of a heterologous protein.

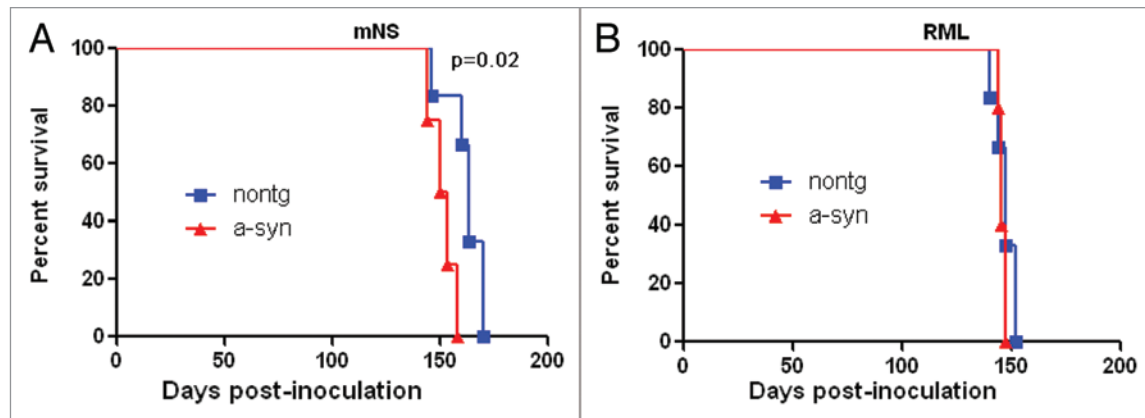
In vitro, small aggregates or fibrils can “seed” or recruit soluble monomers of the same protein and accelerate fibril formation.<sup>7</sup> Cross-seeding experiments further demonstrated that an aggregate nucleus of one protein can induce fibril formation of an unrelated protein.<sup>4,8</sup> Whether cross-seeding occurs in vivo is unclear, however the co-occurrence of distinct protein aggregates indicates the possibility that additional proteins are triggered by

the initial seed to assemble into fibrils.<sup>9,10</sup> A cascade of multiple misfolded proteins may accelerate neurodegeneration and lead to a rapidly progressive clinical disease.<sup>11</sup>

Infectious prions are the only known protein aggregates that naturally transmit to other individuals.<sup>12</sup> In prion disease, the misfolded, aggregated conformer, PrP<sup>Sc</sup>, induces the conversion of the host prion protein, PrP<sup>C</sup>, in an autocatalytic self-templating process.<sup>13</sup> Prion conversion spreads throughout the brain inciting massive astrogliosis, neuronal loss and spongiform degeneration.<sup>14</sup> Once evident, clinical signs are rapidly progressive and ultimately fatal.

With regards to  $\alpha$ -synuclein, protein misfolding and aggregation into protofibrils<sup>15</sup> and oligomers has been shown to lead to neurodegeneration.<sup>16</sup> In addition, recent evidence suggest that prion-like propagation of  $\alpha$ -synuclein from neuron to neuron<sup>17</sup> and neuron to glia<sup>18</sup> might also play an important role in the neurodegenerative process.<sup>19</sup> Although few studies report the co-occurrence of  $\alpha$ -synuclein and prions,<sup>20,21</sup> both proteins are present in synapses,<sup>22,23</sup> and the synaptic loss due to  $\alpha$ -synuclein aggregates may delay or accelerate prion disease. In prion disease, the incubation period is highly predictable and with little variability in lab animal models, which is advantageous in studying modifiers of disease. It is also unclear if prion aggregates

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**Figure 1.** Survival period of prion-infected  $\alpha$ -synuclein transgenic and nontransgenic mice. (A) In mNS-inoculated mice, survival periods were modestly, but significantly accelerated in mice having  $\alpha$ -synuclein aggregates. (B) In contrast, RML-infected mice showed similar survival periods in  $\alpha$ -synuclein transgenic and non-transgenic mice.

can exacerbate  $\alpha$ -synuclein pathology in transgenic models of Parkinson-like disease. In this context, we investigated whether (1) pre-existing human  $\alpha$ -synuclein aggregates modified prion disease pathogenesis or (2) PrP<sup>Sc</sup> exacerbates  $\alpha$ -synuclein pathology. We inoculated infectious prions into aged  $\alpha$ -synuclein transgenic (tg) and non-transgenic (non-tg) littermate control mice by the intracerebral route. Our studies showed that inoculation of RML and mNS prions into  $\alpha$ -synuclein tg mice resulted in more extensive  $\alpha$ -synuclein accumulation with formation of perineuronal  $\alpha$ -synuclein deposits with a neuritic plaque-like appearance.

## Results

**Incubation period for the mNS prion strain is accelerated in  $\alpha$ -synuclein transgenic mice.** We inoculated mouse-adapted natural sheep scrapie (mNS) prions or uninfected brain homogenate into 10-month-old  $\alpha$ -synuclein tg mice and littermate non-transgenic controls to determine whether either disease is modified. In prion-inoculated mice, clinical signs of disease were similar in both  $\alpha$ -synuclein transgenic and nontransgenic mice. However, we found a modest but significant acceleration in the time to terminal prion disease in mice having  $\alpha$ -synuclein aggregates as compared to nontransgenic mice, with incubation periods of  $151 \pm 3$  days as compared to  $162 \pm 4$  days, respectively ( $p = 0.02$ , logrank test) (Fig. 1A). Mice inoculated with uninfected brain homogenate did not develop clinical signs of prion disease.

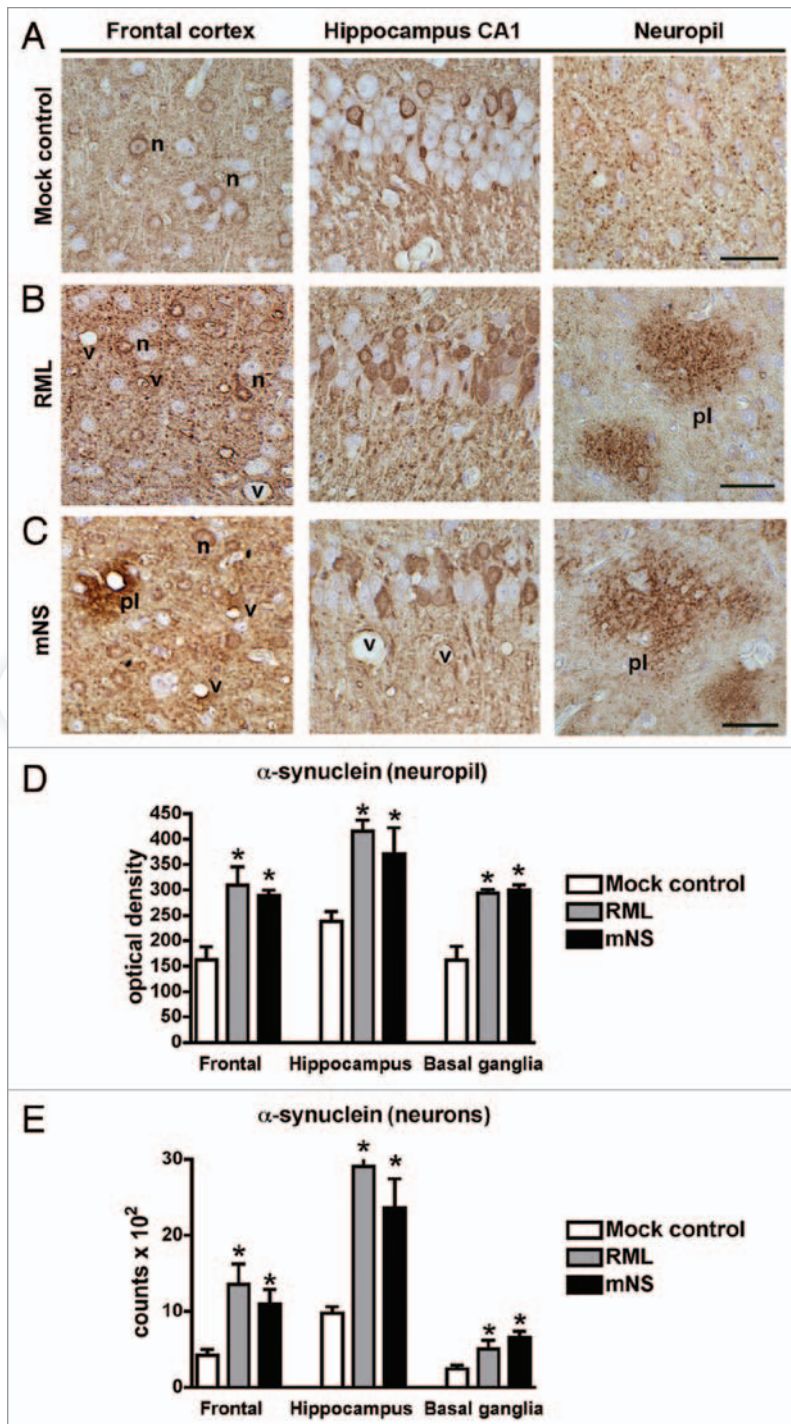
To investigate the possibility of a strain effect in a potential interaction of prions and  $\alpha$ -synuclein, we inoculated mice having  $\alpha$ -synuclein aggregates and controls with a second strain known as Rocky Mountain Laboratory (RML) mouse-adapted prions. In contrast to results with the mNS prions,  $\alpha$ -synuclein mice inoculated with RML prions showed no difference in the prion disease incubation period as compared to control mice (Fig. 1B).

**Inoculation with mNS or RML prions amplifies  $\alpha$ -synuclein pathology in transgenic mice.** To evaluate the effects of the infectious prions on  $\alpha$ -synuclein aggregate morphology and severity, immunocytochemical analysis with a human  $\alpha$ -synuclein

antibody was performed. As expected, in the mock-inoculated tg mice there were abundant  $\alpha$ -synuclein aggregates in the neuropil and neuronal cell bodies in the neocortex, hippocampus, basal ganglia and brainstem (Fig. 2A). In contrast, in the tg mice inoculated with the mNS (Fig. 2B) or RML prions (Fig. 2C) there was more abundant accumulation of  $\alpha$ -synuclein aggregates in the neuropil (Fig. 3D) and neuronal cell bodies (Fig. 3E). Moreover, there was the presence of  $\alpha$ -synuclein immunoreactive plaque-like structures distributed in the neocortex, hippocampus and in the basal ganglia (Fig. 2B and C). Labelled with an antibody against total  $\alpha$ -synuclein, these lesions were 30–60  $\mu$ m in diameter and displayed the presence of abundant peri-neuronal and peri-neuritic  $\alpha$ -synuclein aggregates in the mice inoculated with RML and mNS prions (Fig. 3A). In association with the increased  $\alpha$ -synuclein immunostaining in the neuropil, there were dystrophic neurites of 2–4  $\mu$ m in diameter (Fig. 3A). Comparable dystrophic neuritic profiles were identified with an antibody against phosphorylated  $\alpha$ -synuclein (p-Ser129) (Fig. 3B). Figure 3 illustrates the plaque-like lesions in the basal ganglia, and the lesions observed in the neocortex and hippocampus had a similar structure (Fig. 2B and C).

**Prion pathology is unaltered by the  $\alpha$ -synuclein aggregates.** Next we assessed the histopathology of all prion-infected mice to determine whether the prion-induced pathology was altered by the  $\alpha$ -synuclein. Infection with mNS causes diffuse aggregates and small 5–10  $\mu$ m loose plaques whereas RML prions lead to diffuse plaques in brain. Immunohistochemical stains for PrP<sup>Sc</sup> showed that the mNS prion aggregate morphology did not appear to be altered by the presence of  $\alpha$ -synuclein. Similar to the mNS strain, the RML aggregate morphology was unchanged by the presence of the  $\alpha$ -synuclein aggregates (Fig. 4). Astrogliosis was severe in all prion-infected mice.

Finally we assessed the PrP<sup>Sc</sup> properties by western blotting. We performed a PK-digest and SDS-PAGE followed by immunoblotting for PrP<sup>Sc</sup>. The glycoform profile and electrophoretic mobility of PrP<sup>Sc</sup> were unaltered by the presence of the  $\alpha$ -synuclein (Fig. 5). There was no clear difference in the PrP<sup>Sc</sup> levels in mice with or without  $\alpha$ -synuclein aggregates in the brain.



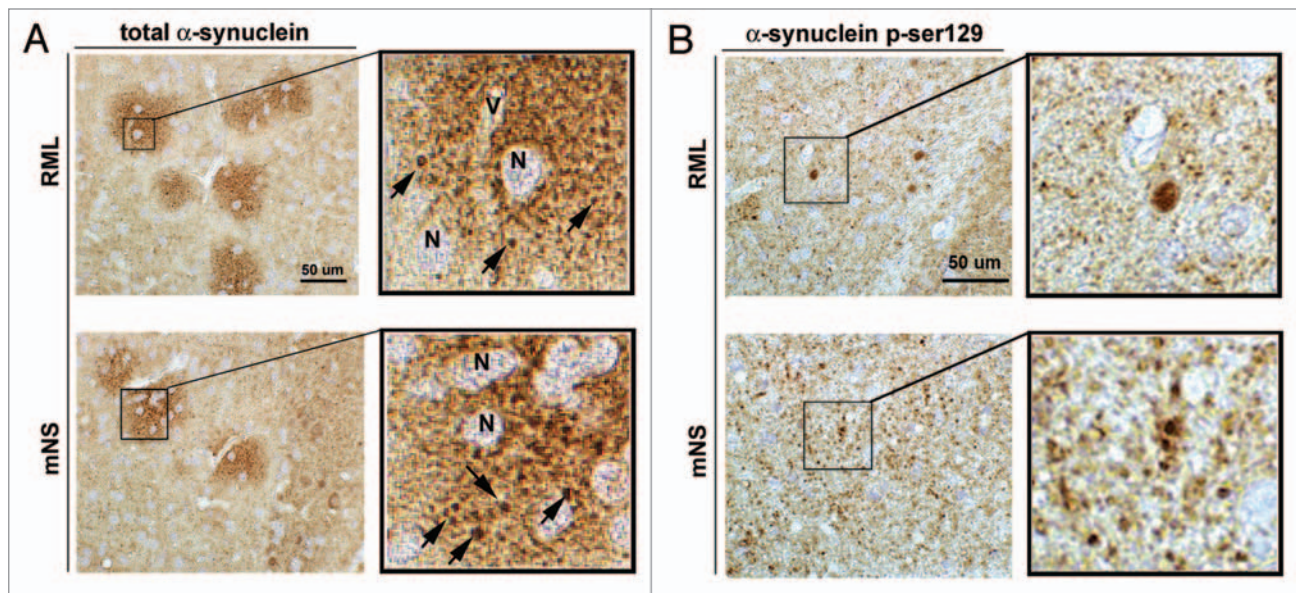
**Figure 2.** Infectious prions promote increased  $\alpha$ -synuclein immunoreactivity. (A)  $\alpha$ -synuclein aggregates typically deposit within neurons (labeled n), shown here in the frontal cortex and hippocampus, and in the neuropil (granular staining) as seen in the mock control. (B) RML prion infection. (C) mNS prion infection. In prion-infected mice,  $\alpha$ -synuclein aggregates were more abundant in neurons and in the neuropil (cortex), and additionally appear as scattered plaque-like structures (labeled pl). Vacuoles due to the prion disease are also evident (labeled v). Scale bar = 20  $\mu$ m. (D) Computer aided image analysis for the levels of  $\alpha$ -synuclein immunoreactivity in the neuropil of the frontal cortex, hippocampus and basal ganglia expressed as corrected optical density. (E) Stereological (dissector method) analysis of the estimated numbers of  $\alpha$ -synuclein positive neurons in the frontal cortex, hippocampus and basal ganglia expressed as total counts  $\times 10^2$ . \* $p < 0.05$  by one way ANOVA with post hoc Dunnett's when comparing to mock control,  $n = 5$  per group.

The present study showed that inoculation of infectious prions into  $\alpha$ -synuclein tg mice exacerbated  $\alpha$ -synuclein pathology and led to  $\alpha$ -synuclein deposits with a neuritic plaque-like appearance. Our study builds on a recent report<sup>24</sup> of prion-infected A53T  $\alpha$ -synuclein tg mice and provides new findings. The study by Mougenot and colleagues shows insoluble, phosphorylated  $\alpha$ -synuclein in uninoculated and H-BSE inoculated mice at terminal disease (14 months of age) by western blot and immunohistochemistry. There was no difference in the severity or characteristics of the  $\alpha$ -synuclein pathology noted. Here we show a remarkable difference in the  $\alpha$ -synuclein pathology in the prion-infected tg mice expressing wildtype  $\alpha$ -synuclein. Consistent with our findings, previous studies also suggest that PrP<sup>Sc</sup> promotes the formation of  $\alpha$ -synuclein lesions. For example, a previous study by Haik and colleagues<sup>20</sup> showed that  $\alpha$ -synuclein immunoreactive deposits accumulate in the brains of patients with sporadic, iatrogenic or variant Creutzfeldt-Jakob disease. In hamsters with experimental scrapie,  $\alpha$ -synuclein accumulated near PrP<sup>Sc</sup> deposits but did not co-localize. Similarly  $\alpha$ -synuclein has been shown to accumulate in the brain of scrapie-affected sheep and goats<sup>21</sup> and occurred in the hippocampus, a site also affected in our prion-infected  $\alpha$ -synuclein mice. Moreover, a recent study by Rodriguez-Diehl and colleagues<sup>25</sup> reported a case of a 64-year-old woman who developed symptoms of sporadic CJD (sCJD). At autopsy, the brain showed classical methionine/methionine PrP<sup>Sc</sup> type 1 (MM1) sCJD changes, moderate Alzheimer-type pathology, as well as  $\alpha$ -synuclein immunoreactive glial cytoplasmic inclusions in the striato-nigral system.

In addition to findings that suggest PrP<sup>Sc</sup> promotes  $\alpha$ -synuclein pathology, previous studies have shown that amyloid  $\beta$  in Alzheimer disease accelerates  $\alpha$ -synuclein pathology<sup>3</sup> in double tg mouse models and in patients with combined Alzheimer and Parkinson disease.<sup>4</sup> Similarly previous reports have shown that Alzheimer disease and CJD can co-exist.<sup>26,27</sup> Together these studies suggest that PrP<sup>Sc</sup> promotes  $\alpha$ -synuclein aggregation and worsens  $\alpha$ -synuclein pathology, and support the notion that misfolded proteins from different neurodegenerative disorders exacerbate disease.

Do preexisting  $\alpha$ -synuclein aggregates modify prion disease progression? In our study, prion pathology was unmodified by the overexpression of  $\alpha$ -synuclein. However, with the mNS prion strain there was a modest but significant acceleration in the time to terminal prion disease in mice having  $\alpha$ -synuclein aggregates as compared to non-tg mice. A recent report showed that tg mice expressing human





**Figure 3.** RML and mNS prions results in the formation of plaque-like  $\alpha$ -synuclein immunoreactive structures. All panels are from the basal ganglia. (A) Immunohistochemical analysis with an antibody against total  $\alpha$ -synuclein. In RML or mNS prion-inoculated tg mice, abundant  $\alpha$ -synuclein aggregates were detected around the neuronal cell bodies (N) and the axonal and dendritic processes. The insets display images at higher power of the  $\alpha$ -synuclein plaque-like structures. The arrows indicate dystrophic neurites in the midst of the plaque-like lesions. (B) Immunohistochemical analysis with an antibody against p-ser129  $\alpha$ -synuclein. In RML or mNS prion-inoculated tg mice, abundant  $\alpha$ -synuclein aggregates were detected in dystrophic neurites. The insets display images at higher power of  $\alpha$ -synuclein affected abnormal axons. Scale bar = 50  $\mu$ m.

A53T  $\alpha$ -synuclein developed accelerated prion disease compared with non-tg controls when inoculated with scrapie, classic BSE or H-type BSE.<sup>24</sup> The histologic and biochemical features of prion disease were unmodified by the A53T  $\alpha$ -synuclein, which was consistent with our findings. The acceleration in this model was greater than the modest effects observed in our study. Such differences may be due to the prion strains used and to the  $\alpha$ -synuclein tg mouse model used. Our mThy1- $\alpha$ -synuclein tg mouse model expresses wild type human  $\alpha$ -synuclein rather than the mutant type,<sup>28</sup> which is known to be more aggressive.<sup>29,30</sup> Further supporting the concept that  $\alpha$ -synuclein does not worsen the prion pathology, a recent study showed that the severity and progression of prion disease was the same in the absence of  $\alpha$ -synuclein.<sup>31</sup> Accumulation of PrP<sup>Sc</sup>, synaptic loss and the behavioural deficits associated with the ME7-agent was the same in wildtype and  $\alpha$ -synuclein knock out mice.

In conclusion, we showed that infectious prions enhance  $\alpha$ -synuclein pathology but not the converse. For future studies, a familial model of prion disease and Parkinson disease would be interesting since  $\alpha$ -synuclein and PrP transit through the ER<sup>32-34</sup> and are present in lipid rafts.<sup>35,36</sup> Important questions that remain to be answered are whether the misfolded PrP<sup>Sc</sup> and  $\alpha$ -synuclein interact in the same intracellular compartment and whether the interactions are indirect.

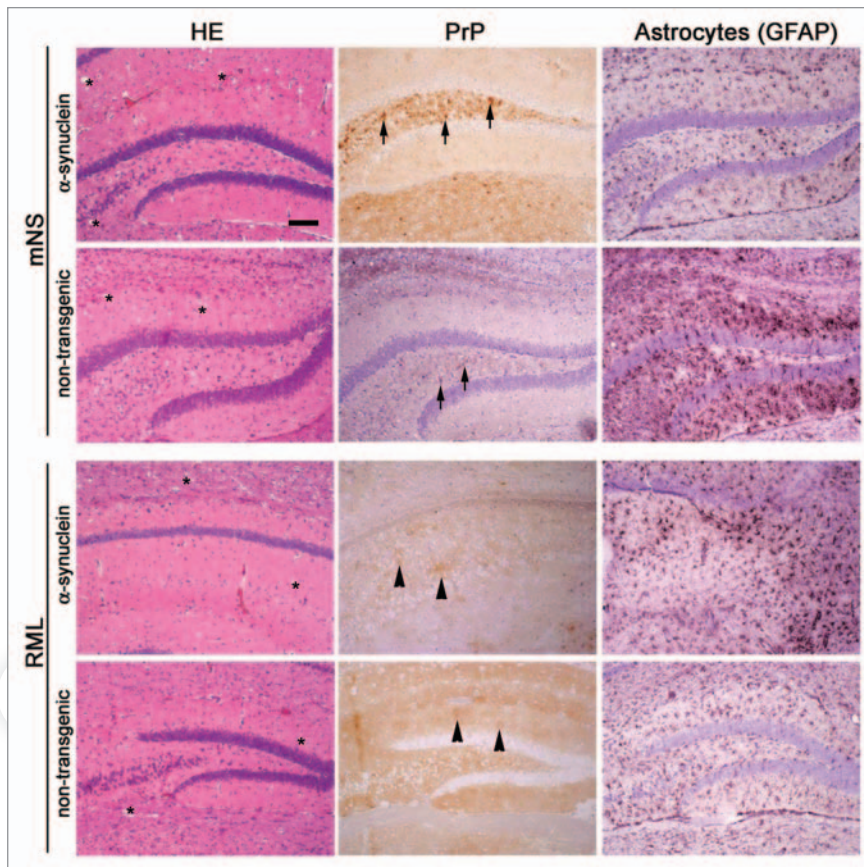
## Methods

**$\alpha$ -synuclein transgenic mice.** All animal procedures were performed in accordance with the protocols approved by the animal care use committee at UCSD following National Institutes of

Health guidelines for the humane treatment of animals. All mice were kept in a normal light/dark cycle (12 hours light/12 hours dark) and had free access to food and water. Mice overexpressing  $\alpha$ -synuclein under a Thy-1 promoter and non-tg littermate controls (C57/BL6 background) were used. The  $\alpha$ -synuclein tg<sup>28,37</sup> mice were previously shown to develop extensive age dependent accumulation of  $\alpha$ -synuclein pathology in the neocortex, hippocampus, basal ganglia and brain stem nuclei accompanied by behavioral deficits.<sup>38</sup>

**Prion inoculations.** Ten-month-old  $\alpha$ -synuclein tg or non-tg littermate control mice (groups of n = 4–6) were intracerebrally inoculated into the left parietal cortex with 30  $\mu$ l of brain homogenate containing RML or mNS mouse-adapted sheep scrapie prions. Uninfected brain homogenate was inoculated into the same mouse genotypes as a negative control. Mice were monitored three times weekly and TSE was diagnosed according to clinical criteria including ataxia, kyphosis, stiff tail, hind leg clasp and hind leg paresis. Mice were sacrificed at the onset of terminal disease or by approximately 185 days post-inoculation and incubation period was calculated from the day of inoculation to the day of terminal clinical disease. Mice were maintained under specific pathogen-free conditions.

**Histopathology and immunohistochemical analysis.** Two-micrometer-thick sections were cut onto positively charged silanized glass slides and stained with hematoxylin and eosin, or immunostained using antibodies for PrP (SAF84) or GFAP for astrocytes. For PrP staining, sections were deparaffinized and incubated for 5 min in 88% formic acid, then washed in water for 5 min, treated with 5  $\mu$ g/ml of proteinase-K, and washed in water for 5 min. Sections were then autoclaved in citrate buffer



**Figure 4.** Prion-induced histopathology is unmodified by the co-occurrence of  $\alpha$ -synuclein aggregates. Brain sections from transgenic ( $\alpha$ -synuclein) and non-transgenic mice inoculated with mNS or RML prions show similar spongiform degeneration (labeled as \* on HE), PrP<sup>Sc</sup> aggregate morphology and astrogliosis. mNS prions induce small (2–4  $\mu$ m), dense, punctate aggregates (arrows), whereas RML prions induce diffuse, patchy aggregates (arrowheads). Scale bar = 200  $\mu$ m.

$\alpha$ -synuclein paraffin sections were incubated with a rabbit polyclonal antibody against total  $\alpha$ -synuclein (Millipore; 1:500) or a mouse monoclonal against pSer129  $\alpha$ -synuclein (Wako, 1:150).

**Computer aided image analysis.** The  $\alpha$ -synuclein immunolabeled blind coded sections were imaged with a digital bright field photomicroscope (Olympus BX41). Captured images (10 per section) from the mock control and prion inoculated  $\alpha$ -synuclein tg mice were delineated and threshold defined and analyzed with the Image Pro-Plus software to determine the corrected optical density value. An additional set of sections counterstained with cresyl violet were analyzed by the dissector method using the Stereo-investigator system to estimate the total number of  $\alpha$ -synuclein immunostained neurons in the frontal cortex, hippocampus and basal ganglia.

**Western blotting for PrP<sup>Sc</sup> in brain.** Samples were electrophoresed in 10% Bis-Tris SDS-PAGE gels (Invitrogen), transferred onto a nitrocellulose membrane and PrP detected using the anti-PrP primary antibody POM1 (epitope in the globular domain, amino acids 121–231, a kind gift from Dr Adriano Aguzzi),<sup>39</sup> and an HRP-conjugated anti-mouse IgG secondary antibody. The blots were developed using a chemiluminescent substrate (ECL detection Kit, Pierce) and visualized on a Fuji LAS 4000 imager. Quantification was performed using Multigauge V3 software (Fujifilm).

(pH 6), cooled for 3 min and washed in distilled water for 2 min. Immunohistochemical stains were performed using the TSA Plus DNP kit (PerkinElmer). Sections were blocked and incubated with anti-PrP SAF-84 (SPI bio; 1:400) for 45 min followed by anti-mouse HRP (Jackson Immunolabs; 1:500) for 30 min. Slides were then incubated with anti-DNP-HRP (PerkinElmer, 1:100) for 30 min, followed by 6 min incubation with DAB. Sections were counterstained with hematoxylin. For detection of

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

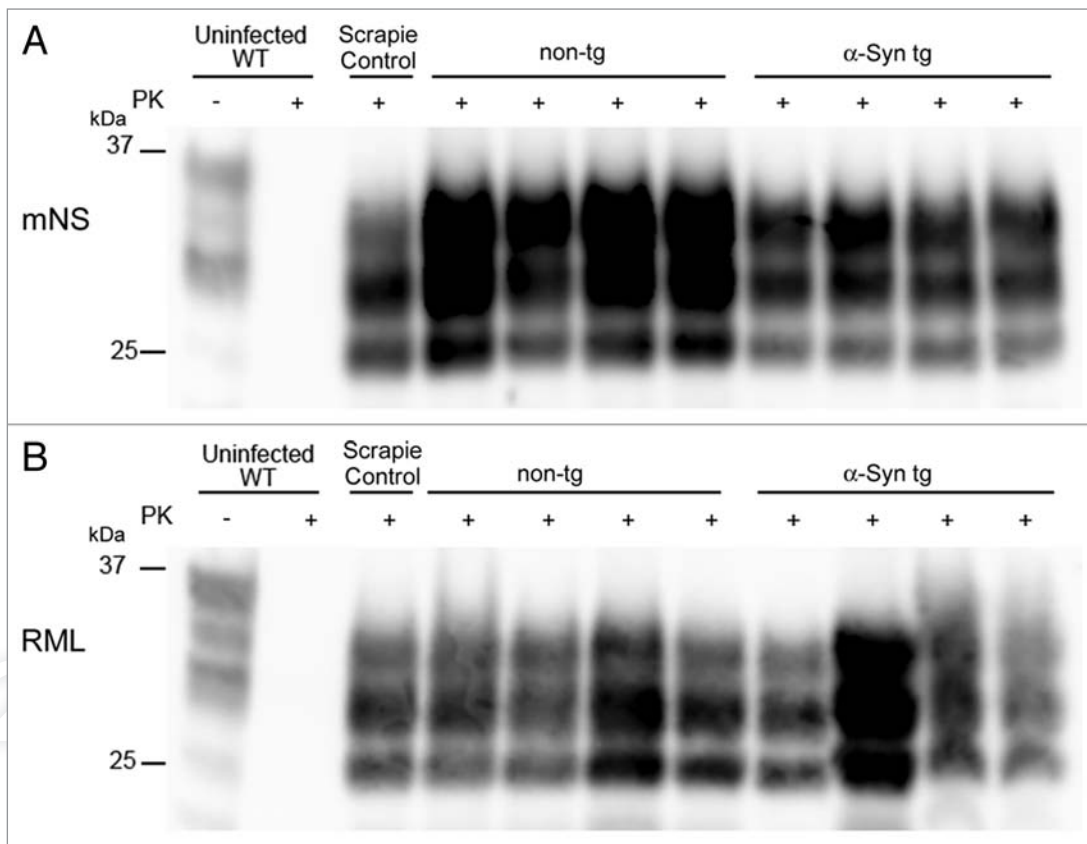
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**Figure 5.** Western blot profiles of PrP<sup>Sc</sup>. A PrP immunoblot for PK-resistant PrP<sup>Sc</sup> reveals similar electrophoretic mobility and glycoform patterns in α-synuclein transgenic and non-transgenic mice inoculated with (A) mNS or (B) RML prions.

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