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Prion Efficiently Replicates in α-Synuclein Knockout Mice

Edoardo Bistaffa[1] · Martina Rossi[2] · Chiara Maria Giulia De Luca[1,2] · Federico Cazzaniga[1] · Olga Carletta[1] · Ilaria Campagnani[1] · Fabrizio Tagliavini[1] · Giuseppe Legname[2] · Giorgio Giaccone[1] · Fabio Moda[1]

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Abstract

Prion diseases are a group of neurodegenerative disorders associated with the conformational conversion of the cellular prion protein (PrP^{C}) into an abnormal misfolded form named PrP^{Sc}. Other than accumulating in the brain, PrP^{Sc} can bind PrP^{C} and force it to change conformation to PrP^{Sc}. The exact mechanism which underlies the process of PrP^{C}/PrP^{Sc} conversion still needs to be defined and many molecules or cofactors might be involved. Several studies have documented an important role of PrP^{C} to act as receptor for abnormally folded forms of α-synuclein which are responsible of a group of diseases known as synucleinopathies. The presence of PrP^{C} was required to promote efficient internalization and spreading of abnormal α-synuclein between cells. In this work, we have assessed whether α-synuclein exerts any role in PrP^{Sc} conversion and propagation either in vitro or in vivo. Indeed, understanding the mechanism of PrP^{C}/PrP^{Sc} conversion and the identification of cofactors involved in this process is crucial for developing new therapeutic strategies. Our results showed that PrP^{Sc} was able to efficiently propagate in the brain of animals even in the absence of α-synuclein thus suggesting that this protein did not act as key modulator of prion propagation. Thus, α-synuclein might take part in this process but is not specifically required for sustaining prion conversion and propagation.

Keywords Prions · RML · α-Synuclein · PMCA

Introduction

Prion diseases are a group of incurable neurodegenerative conditions associated with the accumulation in the central nervous system (CNS) of an abnormally folded and pathological form of the cellular prion protein (PrP^{C}), named prion (PrP^{Sc}) [1, 2]. PrP^{C} is soluble in mild detergents, sensitive to proteases digestion and contains two N-linked glycosylation sites (at asparagine residues 181 and 197) which give rise to un-glycosylated, mono-glycosilated, and di-glycosylated PrP species (referred to as PrP glycoform ratio) [3]. In contrast, PrP^{Sc} is rich in β-sheet structures, is partially resistant to proteases

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✉ Fabio Moda
fabio.moda@istituto-besta.it

Edoardo Bistaffa
edoardo.bistaffa@istituto-besta.it

Martina Rossi
martina.rossi@sissa.it

Chiara Maria Giulia De Luca
chiara.deluca@istituto-besta.it

Federico Cazzaniga
federico.cazzaniga@istituto-besta.it

Olga Carletta
olga.carletta01@universitadipavia.it

Ilaria Campagnani
ilarialaveterinaria@libero.it

Fabrizio Tagliavini
fabrizio.tagliavini@istituto-besta.it

Giuseppe Legname
legname@sissa.it

Giorgio Giaccone
giorgio.giaccone@istituto-besta.it

[1] Unit of Neuropathology and Neurology 5, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy

[2] Department of Neuroscience, Laboratory of Prion Biology, Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste, Italy

degradation and is almost insoluble in detergent. Above all, PrP^{Sc} coerces PrP^{C} to adopt the PrP^{Sc} conformation, thus contributing to the spreading of prion pathology throughout the brain [4–8]. After proteinase K (PK) digestion, PrP^{C} is completely degraded while PrP^{Sc} shows a PK-resistant, C-terminal core of 27–30 kDa. PrP^{Sc} might acquire different abnormal conformations (referred to as prion strains) that are able to induce diseases characterized by different clinical and neuropathological phenotypes. From a biochemical point of view, each prion strain is characterized by its own (i) glycoform ratio (after PK-digestion), (ii) electrophoretic mobility of the un-glycosylated PrP isoform, (iii) resistance to PK digestion, and (iv) stability against guanidine hydrochloride (Gdn-HCl) treatment [7, 9–11]. Neuropathologically, each strain induces peculiar (i) clinical manifestations, (ii) spongiform changes, and (iii) patterns of PrP^{Sc} deposition in different brain regions [12–17]. The exact mechanism which leads to PrP^{C}-PrP^{Sc} conversion is still not well understood, as well as the mechanisms through which PrP^{Sc} spreads in the CNS [18]. Different mechanisms of PrP^{Sc} spreading have been proposed including the formation of exosomes, nanotubes, or cell-to-cell contacts, and most probably, all of them contribute to some extent to prion propagation [19–23]. Nevertheless, PrP^{Sc} could spread even "naked" in the CNS using other proteins as receptors. In particular, the role of α-synuclein as possible receptor for PrP^{Sc} or modulator of prion diseases is now under investigation. α-Synuclein is highly expressed in the brain [24], especially at nuclear and synaptic terminals [25, 26], and is involved in many cellular functions (including neuronal differentiation [27, 28], dopamine biosynthesis [29], neurotransmitter release [30], and vesicles trafficking [31]). Among these functions, the flexible conformation of α-synuclein suggests that it can act as chaperone or eventually cooperate with other chaperones (e.g., Hsp70 and Hsp40) or co-chaperones (e.g., cysteine string protein-α and small glutamine-rich TPR protein) for enabling an efficient neurotransmitter release and, eventually, for maintaining protein homeostasis in neuronal cells [32–34]. Compelling evidences indicated that α-synuclein possesses antioxidant effects and exerts an important role in protecting against neurodegeneration [35–37]. For these reasons, modification of α-synuclein functions might lead to neurodegeneration. This protein is found abnormally folded in a group of neurodegenerative disorders known as synucleinopathies which includes Parkinson's disease (PD) [38], dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) [38, 39]. As for PrP^{Sc}, abnormally folded forms of α-synuclein can recruit and convert their physiological counterparts thus contributing to the spreading of α-synuclein pathology either in vitro or in vivo [40–44]. However, as in the case of PrP^{Sc}, the mechanisms of α-synuclein spreading remain unknown. We and others have recently demonstrated that PrP^{C} acts as receptor for abnormally folded forms of α-synuclein allowing their efficient internalization in cells [45, 46]. Moreover, we have observed that the presence of α-synuclein aggregates in prion-infected cells lines were able to decrease the levels of infectivity upon passages. Probably, the interaction between α-synuclein aggregates and PrP^{C} on the cell surface efficiently blocked PrP^{C}-PrP^{Sc} interaction and conformational conversion [45].

In a very recent paper, Baskakov and collaborators [47] have demonstrated that abnormally folded α-synuclein interacts with PrP^{C} and forces it to acquire a pathological conformation in vitro. When injected in mice, this new PrP species induced prion diseases upon serial passages in wild-type animals. Taken together, these data strongly support the evidence that an interplay between α-synuclein and PrP^{C} exists and might play a role in the spreading of these abnormally folded proteins in the CNS. As previously indicated, many studies have demonstrated the ability of PrP^{C} to act as receptor for abnormally folded forms of α-synuclein.

In this study, we have assessed the opposite event, that is the role of α-synuclein to act either as receptor for PrP^{Sc} or as disease modulator in mice injected with RML prion strain. Moreover, we have exploited the protein misfolding cyclic amplification (PMCA) technique to evaluate the ability of RML to replicate in vitro in the absence of α-synuclein. Finally, we have evaluated whether lack of α-synuclein could have modified the biochemical properties of RML either in vitro or in vivo. Indeed, recent evidences have demonstrated that modifications of the replication environment play an important role in prion selection and adaptation [48–51]. Therefore, the lacking of α-synuclein could have modified the replication environment resulting in modifications of the biochemical features of RML.

Knowing whether α-synuclein plays a role in the pathogenesis of prion diseases could have important applications for identifying innovative therapeutic approaches for these devastating disorders.

Materials and Methods

Ethics Statement

C57BL/6JOlaHsd and C57BL/6JRccHsd mice were purchased from Harlan Laboratories UK. Animals were housed in individually ventilated cages (2–5 mice per cage), daily fed, and provided with water. Lighting was on an automatic 12 h basis. Regular care was periodically performed for assessment of animal health. Animal facility is licensed and inspected by the Italian Ministry of Health. Current animal husbandry and housing practices comply with the Council of Europe Convention ETS123 (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes; Strasbourg, 18.03.1986), Italian

Legislative Decree 116/92 (Gazzetta Ufficiale della Repubblica Italiana, 18th February 1992), and with the 86/609/EEC (Council Directive of 24 November 1986 on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes). The study, including its Ethics aspects, was approved by the Italian Ministry of Health (Permit Number: NP-02-13), and all efforts were made to minimize animals suffering.

Mouse Models

C57BL/6JOlaHsd mice (α-syn KO) are characterized by a spontaneous depletion of α-synuclein gene (*SNCA* knock out) without any compensatory effect due to the up-regulation of β- or γ-synuclein [52]. C57BL/6JRccHsd mice (WT) represent their wild-type controls.

Intracerebral Injection of RML or Mock Brain Homogenates

Ten percent (weight/volume (*w*/*v*)) RML brain homogenate was prepared in sterile PBS and diluted at 10^{-3} (volume/volume (*v*/*v*)) in the same buffer. Six-week-old α-syn KO (35–40 g) were anesthetized with tribromoethanol (100 μL/10 g) and stereotaxically injected in the hippocampus (1.8 caudal; + 0.5 lateral; 1.8 depth) with 2 μL of RML ($n = 5$) or Mock ($n = 5$) brain homogenates. Similarly, WT mice were injected with RML ($n = 5$) or Mock ($n = 5$) brain homogenates. All surgical procedures were performed under sterile conditions.

Incubation and Survival Time

The incubation time (IT) was calculated considering the time between RML injection and symptoms onset, including ataxia (uncoordinated movement), tail rigidity, and kyphosis (hunched back). Survival time (ST) was calculated as the time between RML injection and the killing of animals. Mice injected with Mock were culled along with terminally sick RML-injected animals and were used as negative controls. Brains were then harvested and divided in two hemispheres. The right hemisphere was used for neuropathological assessments while the left one was used for biochemical analysis.

Neuropathological Assessments

The right hemispheres of the brain were fixed in Alcolin (Diapath), dehydrated, and embedded in paraffin (Bio-Optica). Serial sections (7 μm) were cut and stained with hematoxylin-eosin (H&E) or immunostained with monoclonal antibodies to PrP (6H4, Prionics) or α-synuclein (4D6, Abcam) and polyclonal antibody to GFAP (Dako). Before PrP immunostaining, sections were treated with Proteinase K (10 μg/mL, 5 min, room temperature, Invitrogen) and guanidine isothiocyanate (3 M, 20 min, room temperature, Carlo Erba), while before GFAP immunostaining, sections were treated with 10% formalin (1 h, room temperature). Unspecific binding of the secondary antibody was prevented using the animal research kit (ARK, Dako). Immunoreactions were visualized using 3–3′-diaminobenzidine (DAB, Dako) as substrate-chromogen system. Lesion profile was performed on H&E stained sections according to Fraser et al. [15]. The following brain areas were evaluated: (1) dorsal medulla, (2) cerebellar cortex, (3) superior culliculus, (4) hypothalamus, (5) thalamus, (6) hippocampus, (7) septum, (8) retrosplenial and adjacent motor cortex, and (9) cingulated and adjacent motor cortex. For each brain area, the severity of vacuolar changes was scored from 0 (no lesions) to 3 (extensive vacuolization). All images were acquired with Nikon Eclipse E800 microscope equipped with Nikon digital camera DXM 1200 and Nikon ACT-1 (v2.63) acquisition software.

Biochemical Analysis

The left hemispheres of the brains were homogenized at 10% (*w*/*v*) in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% NP40, 0.5% Na-deoxycholate, 10 mM Tris-HCl pH 7.4) and centrifuged 800×*g* for 1 min. Twenty microliters of cleared lysates was supplemented with loading buffer (sample buffer 4× and DTT 10×, Thermo Scientific) and boiled (100 °C, 10 min). Proteins were fractionated using 12% Bis-Tris Plus gels (Thermo Scientific) and transferred onto polyvinylidene difluoride membranes (PVDF, Millipore). Before PrP^{Sc} analysis, samples were treated with PK 50 μg/mL (see "PK Digestion" for details). After blocking with non-fat dry milk (1 h, room temperature), the membranes were incubated with monoclonal antibody to PrP (6D11, 0.2 μg/mL, Covance), monoclonal antibody to α-synuclein (4D6, 0.2 μg/mL, Abcam), or monoclonal antibody to α-tubulin (clone T9026, 0.2 μg/mL, Sigma). Blots were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (GE) and developed using the ECL Prime detection system (Amersham). Chemiluminescence was visualized using a G:BOX Chemi Syngene system.

PK Digestion

Twenty microliters of cleared brain homogenates was treated with 50 μg/mL of PK under shaking (1 h, 37 °C, 550 rpm, Invitrogen). Enzymatic digestion was stopped by boiling (100 °C, 10 min) the samples supplemented with loading buffer (sample buffer 4× and reducing agent 10×, Thermo Scientific) and Western blot analysis were performed as previously described.

PK Resistance Assay

Twenty microliters of brain homogenates was treated with five increasing concentrations of PK (50, 100, 250, 500, and 1000 μg/mL) for 1 h at 37 °C under continuous shaking. Samples were then immunoblotted with 6D11 antibody and densitometric analysis was performed.

PMCA Procedures

PMCA was performed as previously described [53]. Briefly, brains of α-syn KO or WT mice were homogenized at 10% (*w*/*v*) in conversion buffer (150 mM sodium chloride, 1% Triton X-100 in PBS 1×), supplemented with complete protease inhibitor cocktail (Roche) and used as PMCA substrates. RML and Mock brain homogenates were serially diluted (from 10^{-5} to 10^{-8}) and 10 μL of each dilution was added to 90 μL of each PMCA substrate. Samples were then subjected to PMCA analysis performed by alternating cycles of sonication (20 s, 250–260 W) to cycles of incubation (29 min 40 s) at 37–40 °C, using a micro-sonicator (Misonix, S3000). To increase the efficiency of amplification, 3 teflon beads were added to each sample. After 96 cycles (referred to as PMCA round), 20 μL of the amplified products was subjected to Western blot analysis.

Statistical Analysis

Statistical analysis was performed using the Prism software (v5.0 GraphPad). Kaplan-Meier survival curves were plotted and differences in incubation and survival times between groups of animals were compared using the Logrank test. Densitometric analysis of Western blot bands was performed using ImageJ software (1.51v). Graphic representations of densitometric analysis and lesion profile were performed using the Prism software (v5.0 GraphPad).

Results

PrP^C Expression Levels Are Similar in Both α-Syn KO and WT Mice

Brain homogenates of α-syn KO and WT mice were analyzed by means of Western blot in order to evaluate whether the lack of α-synuclein might have affected the total expression level of PrP^C (Fig. S1a). To this aim, samples were immunblotted either with an antibody against PrP^C (6D11) or with an antibody against α-tubulin. The intensity of PrP^C signal was then normalized against that of α-tubulin. Results indicated that the expression levels of PrP^C were identical between α-syn KO and WT mice. The lack of α-synuclein in KO mice was confirmed either with immunohistochemical (Fig. S1b) or biochemical analysis (Fig. S1c).

RML Efficiently Amplifies in α-Syn KO and WT Brain Homogenates by Means of PMCA

Serial dilutions of RML and Mock brain homogenates (from 10^{-5} to 10^{-8}, *v*/*v*) were prepared and subjected to PMCA using both α-syn KO and WT brain homogenates as substrates of reaction. One round of amplification was enough to amplify with high efficiency all RML dilutions in both α-syn KO and WT brain homogenates (Fig. 1a). As expected, we did not observe any prion amplification in samples supplemented with Mock dilutions (Fig. 1b). These data indicate that α-synuclein was not necessary for an efficient RML amplification in vitro. However, PMCA represents an artificial environment which lacks of specific interactions which occur in vivo. Therefore, we have carried out in vivo experiments by inoculating α-syn KO and WT mice with RML prion strain in order to better analyze any eventual role of α-synuclein to function as receptor for PrP^{Sc}.

RML Efficiently Replicates in α-Syn KO and WT Mice

To assess the potential role of α-synuclein to interact with PrP^{Sc} while promoting its cellular internalization and spreading throughout the CNS, we have performed in vivo experiments. Particularly, 2 μL of either RML or Mock brain homogenates were diluted at 10^{-3} (*v*/*v*) and inoculated in the hippocampus of 6-week-old α-syn KO and WT mice. Regardless of the genetic background (α-syn KO vs. WT), all the animals developed prion pathology with similar incubation (α-syn KO 142.40 ± 5.24 and WT 144.60 ± 2.44) and survival time (α-syn KO 182.40 ± 4.50 and WT 185.75 ± 2.50) (Fig. 2a). Log-rank test analysis confirmed that the incubation ($p = 0.6683$) and survival time ($p = 0.5775$) between groups did not reach statistically significant differences. None of the animals injected with Mock brain homogenate developed prion pathology but were sacrificed along with RML-injected animals and were used as negative controls.

α-Syn KO and WT Mice Injected with RML Show Similar Neuropathological Alterations

Neuropathological analysis revealed that all RML injected animals showed similar alterations, independently on their genetic background (α-syn KO vs. WT). Particularly, spongiform changes mainly affected the thalamus, hippocampus, and frontal cortex while dorsal medulla, cerebellum, and hypothalamus were less affected (Figs. 2b and 3a). Immunohistochemical analysis revealed typical pattern of RML-PrP^{Sc} accumulation that was mainly synaptic and diffuse throughout the brain with strong immunoreaction

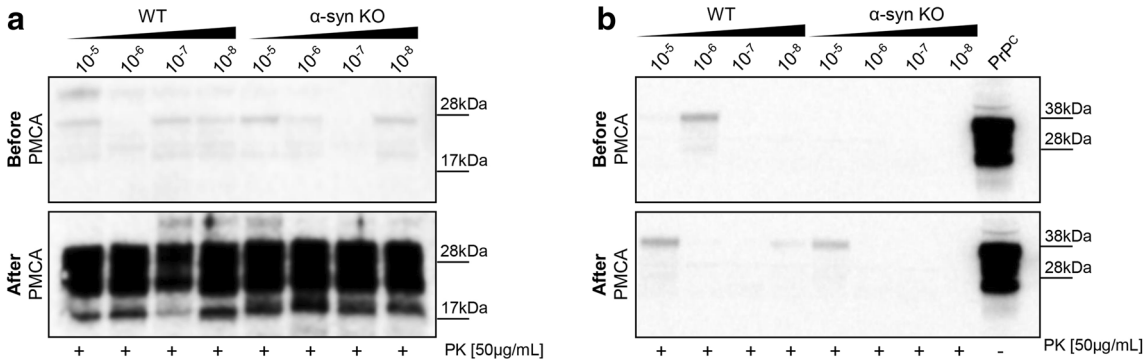

Fig. 1 PMCA of RML prion strain. **a** Amplification of RML using α-syn KO and WT brain homogenates as reaction's substrates. RML brain homogenate was spiked (from 10^{-5} to 10^{-8}, *v/v*) in α-syn KO and WT brain homogenates and subjected to PMCA analysis. After one round of PMCA, all dilutions were efficiently amplified with both substrates. After PK digestion and Western blot analysis, the membranes were immunostained with anti-PrP antibody (6D11). **b** Amplification of Mock using α-syn KO and WT brain homogenates as reaction's substrates. The same dilutions of Mock brain homogenate were spiked in α-syn KO and WT substrates and subjected to PMCA. PK digestion and Western blot analysis confirmed the lack of PrP^{Sc} signals. PrP^{C} lane refers to normal mouse brain homogenate. Numbers in the right indicate the position of molecular weights

detected in the hippocampus, thalamus, and frontal cortex (Fig. 3a). The severity of GFAP activation was found to correlate with PrP^{Sc} accumulation and was therefore higher in the hippocampus, thalamus and frontal cortex than that observed in other brain regions (Fig. 3a). These results indicate that RML-PrP^{Sc} was able to indiscriminately propagate in both α-syn KO or WT mice while producing comparable neuropathological alterations. Thus, the lack of α-synuclein seemed not to modify the infectious and neuropathological features associated with RML-PrP^{Sc}. Mock-injected animals did not show spongiform changes or PrP^{Sc} deposition in the brain while physiological GFAP activation was detected (Fig. 3b).

Similar PK-Resistant PrP^{Sc} Is Found in the Brain of RML Injected α-Syn KO and WT Mice

Brain homogenates of α-syn KO and WT mice injected with RML were treated with PK and analyzed by means of Western

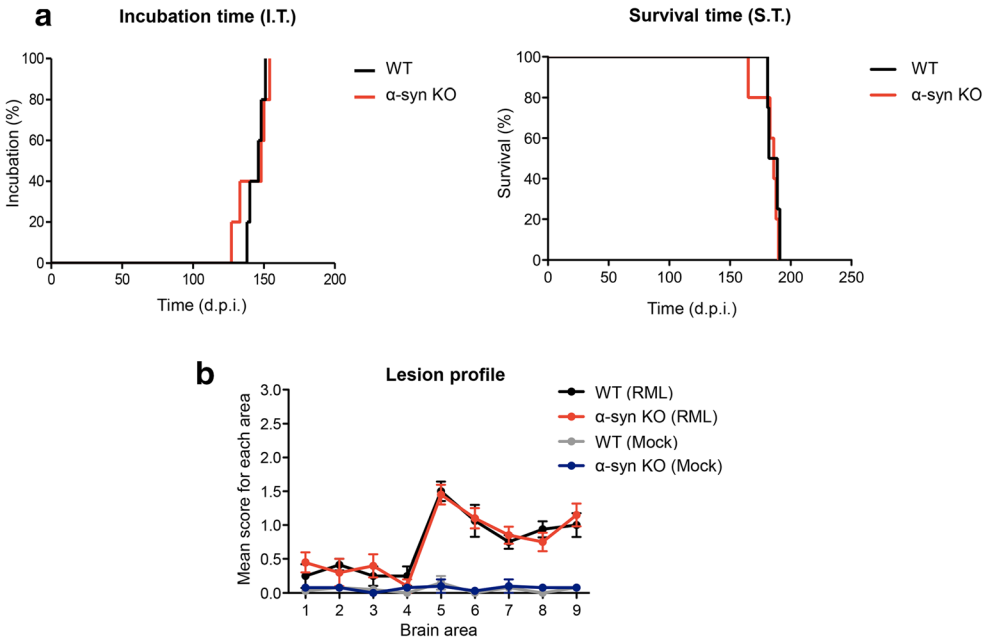

Fig. 2 Incubation time, survival time, and lesion profile of RML-injected animals. **a** Incubation (IT) and survival (ST) times of α-syn KO and WT mice injected with RML. Incubation and survival times were not statistically different between α-syn KO and WT mice injected with RML (IT $p = 0.6683$; ST $p = 0.5775$; Log-rank test). **b** Lesion profile of α-syn KO and WT mice injected with RML prion strain. The spongiform alterations were evaluated on H&E-stained sections in nine standard brain areas (1 = dorsal medulla, 2 = cerebellar cortex, 3 = superior colliculus, 4 = hypotalamus, 5 = thalamus, 6 = hippocampus, 7 = septum, 8 = retrosplenial and adjacent motor cortex, 9 = cingulated and adjacent motor cortex). All groups of RML-injected mice showed similar spongiform alterations while all Mock-injected animals did not show spongiosis. Error bars represent the standard error of the mean (mean ± SEM)

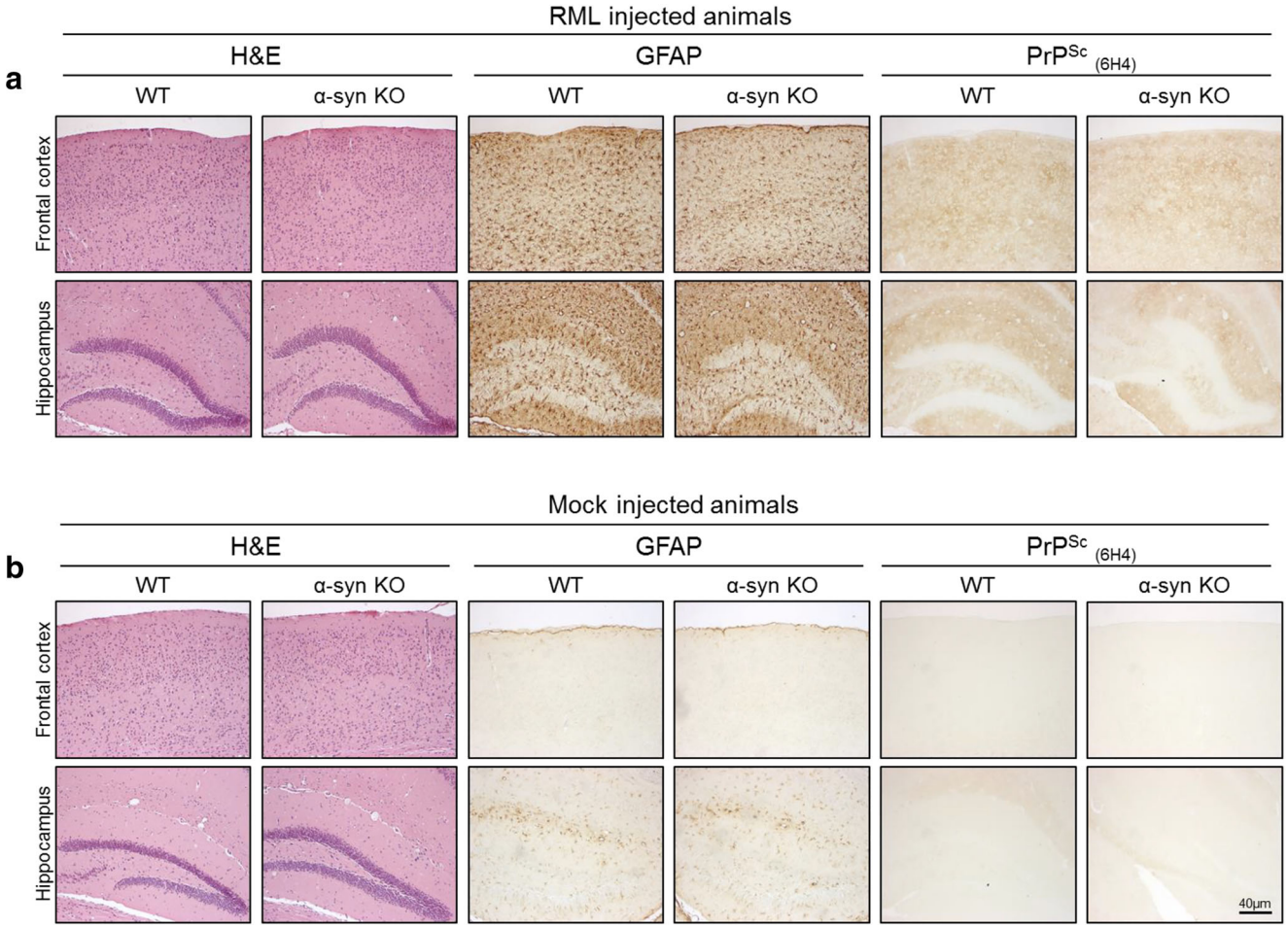

Fig. 3 Neuropathological alterations of RML injected animals. **a** Neuropathological analysis of RML-injected animals. Immunohistochemical analysis showed no differences in PrP^{Sc} deposition between α-syn KO and WT RML-injected mice. Glial activation was found to correlate with PrP^{Sc} accumulation and confirmed the lack of differences among groups of animals. **b** Neuropathological analysis of Mock-injected animals. No PrP^{Sc} deposition or abnormal glial activation were found in the brain of Mock injected animals. Scale bar, 40 μm

blot. PK-resistant PrP^{Sc} was detected in all RML injected animals and its biochemical properties, including glycoform ratio and migration of the three PrP species, were similar in α-syn KO and WT mice. The typical RML associated PrP^{Sc} characterized by the prevalence of the mono-glycosylated PrP species with the unglycosylated ones migrating at around 21 kDa (Fig. 4a) was detected in both groups of animals.

PK-Resistant PrP^{Sc} of RML Injected in α-Syn KO and WT Mice Retains the Same Biochemical Features in Both Groups of Mice

It is known that modifications of the replication environment can modify either the infectious or the biochemical properties of prion stains. To evaluate whether the lack of α-synuclein might have influenced the biochemical properties of RML, we have performed a PK-resistance assay. To this aim, brain homogenates of α-syn KO and WT mice injected with RML were treated with increasing concentrations of PK (from 50 μg/mL to 1 mg/mL). Samples were then analyzed by means of Western blot and densitometric quantification of the PK-resistant PrP signals were then performed (Fig. 4b). This analysis confirmed that the biochemical features of RML-PrP^{Sc} in α-syn KO were similar to those of WT mice.

Discussion

In this work, we have assessed the effects that the spontaneous depletion of α-synuclein in the brain of C57BL/6JOlaHsd mice (referred to as α-syn KO animals) could have had on the infectious and neuropathological features of RML prion strain. Although lacking the expression of α-synuclein, these mice do not develop spontaneous neurodegeneration [52, 54, 55] and the PrP^{C} expression levels are similar to those of control (referred to as WT) animals. Therefore, any disease modification could not be associated with the reduction of the substrate required for prion propagation. Compelling

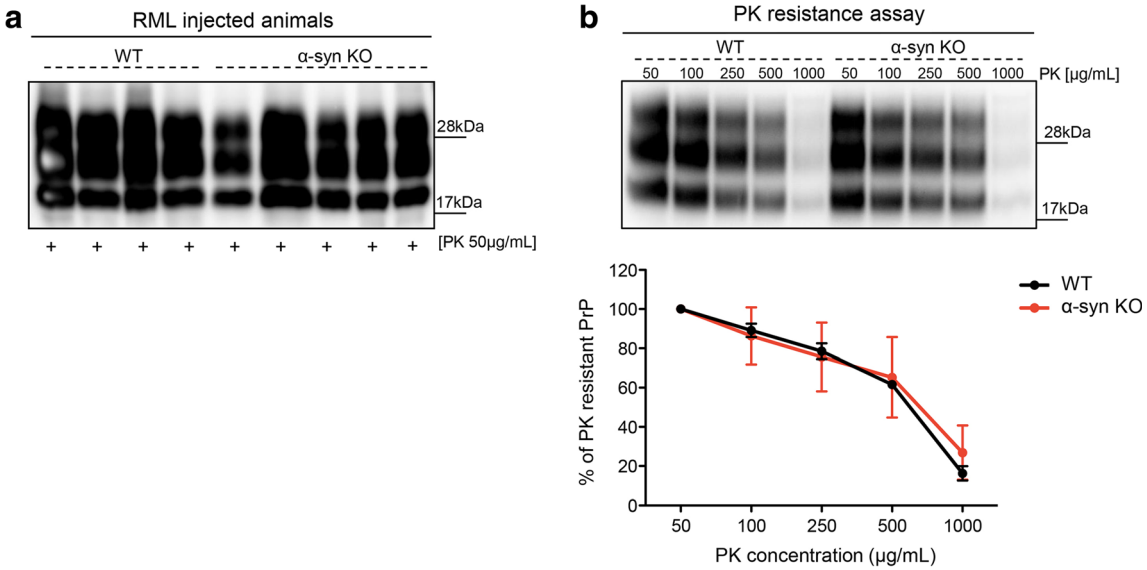

Fig. 4 Biochemical features of RML-PrP^{Sc} present in the brain of α-syn KO and WT mice. **a** Western blot analysis. Biochemical analysis of brain homogenates of RML-injected animals showed similar levels of PrP^{Sc} between α-syn KO and WT animals. **b** PK resistance assay. Brain homogenates of WT and α-syn KO RML-injected mice were treated with increasing concentration of PK (50, 100, 250, 500, and 1000 μg/mL) and then analyzed by means of Western blotting. Densitometric analysis of PK-resistant PrP signals confirmed the lack of statistically significant differences between groups of animal. Error bars represent the standard error of the mean (mean ± SEM). Numbers in the right indicate the position of molecular weights

evidences suggest that α-synuclein acts as chaperone and antioxidant protein [56], hence we have decided to verify whether this protein could interact with PrP^{C} or PrP^{Sc} thus modulating the course of prion diseases.

As we previously reported [45], PrP^{C} is an important receptor for α-synuclein amyloid fibrils. The presence of PrP^{C} was found to be fundamental for the internalization of α-synuclein amyloids in cells and interaction between α-synuclein and PrP^{C} efficiently reduced the replication of RML prion strain in cell cultures. Based on these observations, we decided to assess whether physiological α-synuclein could function as interactor for PrP^{Sc} or exert any role in prion propagation. First of all, through PMCA analysis we showed that RML-PrP^{Sc} efficiently replicates even in substrates depleted of α-synuclein (brain homogenates of α-syn KO mice). This was an initial evidence suggesting that α-synuclein was not likely to be necessary for sustaining an efficient RML-PrP^{Sc} replication. However, PMCA provides only a permissive molecular context for prion replication but does not recapitulate the cellular environment which is instead characterized by specific cell-to-cell interactions, subcellular compartments (e.g., lysosomes and endosomes) or active proteases activity (the substrates were prepared in buffers containing protease inhibitors). For these reasons, any interaction between α-synuclein and PrP^{Sc} might have not been properly occurred. Therefore, we have decided to perform the experiments in vivo by injecting RML-PrP^{Sc} prion strain in the brain of α-syn KO and WT mice. In this way, we have performed the experiments in a context where all the physiological conditions eventually required for the interaction between PrP^{Sc} and α-synuclein could have occurred. All RML-infected animals succumbed to prion disease with similar incubation and survival times, independently on their genetic background. The severity of spongiform changes and glial activation was comparable between α-syn KO and WT mice and both groups of animals showed abundant PrP^{Sc} accumulation, characterized either by the typical RML biochemical features (in terms of glycoform ratio and resistance to increasing concentrations of PK) or pattern of deposition in the brain. These results supported our in vitro observations, indicating that the lack of α-synuclein did not substantially modulate prion propagation and the pathological features associated with RML prion strain in vivo. In particular, the absence of α-synuclein and its putative chaperone activity did not produce prion disease worse than that of WT animals. Probably, many other cellular chaperones cooperate in compensating the lack of folding modulator role of α-synuclein [57]. Indeed, it has been shown that alterations in α-synuclein expression levels led to the up-regulation of specific chaperones, including Hsp27, Hsp40, Hsp70, and caspase 9 in vivo [58]. Nevertheless, the function of other synaptic proteins, including the class-A2 lipid-binding proteins, that share similar characteristics with α-synuclein, are likely to sustain prion propagation. In particular, such proteins have been shown to interact with PrP^{Sc} in vitro *or* in vivo [59–63] and many of them, including the apolipoprotein E were found to co-purify with PrP^{Sc} collected from different mouse and hamster prion strains [64]. These data suggest that synaptic proteins might

effectively interact with PrP^{Sc} eventually contributing to its spreading in the central nervous system. Thus, a combination of proteins, including α-synuclein, might function as PrP^{Sc} interactors and play a role in prion propagation. For this reason, specific α-synuclein depletion might not have significantly affected the course of prion propagation, thus indicating that more complex mechanisms are associated with this process.

Another important aspect which needs to be taken into account is the conformational effect which suggests that different abnormal conformations of PrP^{Sc} might have distinct propensities to interact with α-synuclein. For instance, the presence of both PrP^{Sc} and α-synuclein aggregates in the brain of the same patient have been reported only in few cases of human prion diseases [65, 66] thus highlighting the fact that α-synuclein might selectively interact with specific prion strains. Particularly, α-synuclein aggregates were found in the brain of few patients with prion diseases characterized by longer duration [67]. This phenomenon was found to occasionally occur also in animals where granular deposits of α-synuclein were observed in brains of sheep and goat affected by natural scrapie [68]. Taken together, these data clearly suggest that α-synuclein and PrP^{Sc} amyloids might somehow interact in a way that still need to be understood.

In our experimental conditions, RML-PrP^{Sc} was able to propagate even in the absence of α-synuclein either because this protein is not really required for its replication (as recently reported [69]) or because many other factors, proteins and molecular mechanisms might cooperate in promoting prion replication. Defining the role of α-synuclein in prion diseases is of fundamental importance for developing novel therapeutic strategies for these devastating disorders.

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Author Contributions E.B. performed most of the experiments; M.R., C.D.L., performed part of the biochemical analysis; F.C., O.C. performed part of the histological analysis; I.C. was in charge of the animal care and sacrifice; F.T. and G.L. contributed in planning the experiments and in analyzing the data; and G.G. and F.M. supervised the work, analyzed the data and prepared the final version of the manuscript. All the authors read and approved the final manuscript.

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Compliance with Ethical Standards

The study, including its Ethics aspects, was approved by the Italian Ministry of Health (Permit Number, NP-02-13).

Conflict of Interest The authors declare that they have no conflict of interest.

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