RESEARCH ARTICLE

Cellular Prion Protein Mediates α-Synuclein Uptake, Localization, and Toxicity In Vitro and In Vivo

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ABSTRACT: Background: The cellular prion protein (PrP^C) is a membrane-bound, multifunctional protein mainly expressed in neuronal tissues. Recent studies indicate that the native trafficking of PrP^C can be misused to internalize misfolded amyloid beta and α-synuclein (aSyn) oligomers.
Objectives: We define PrP^C's role in internalizing misfolded aSyn in α-synucleinopathies and identify further involved proteins.
Methods: We performed comprehensive behavioral studies on four transgenic mouse models (ThySyn and ThySynPrP00, TgM83 and TgMPrP00) at different ages. We developed PrP^C-(over)-expressing cell models (cell line and primary cortical neurons), used confocal laser microscopy to perform colocalization studies, applied mass spectrometry to identify interactomes, and determined disassociation constants using surface plasmon resonance (SPR) spectroscopy.
Results: Behavioral deficits (memory, anxiety, locomotion, etc.), reduced lifespans, and higher oligomeric aSyn levels were observed in PrP^C-expressing mice (ThySyn and TgM83), but not in homologous *Prnp* ablated mice (ThySynPrP00 and TgMPrP00). PrP^C colocalized with and facilitated aSyn (oligomeric and monomeric) internalization in our cell-based models. Glimepiride treatment of PrP^C-overexpressing cells reduced aSyn internalization in a dose-dependent manner. SPR analysis showed that the binding affinity of PrP^C to monomeric aSyn was lower than to oligomeric aSyn. Mass spectrometry-based proteomic studies identified clathrin in the immunoprecipitates of PrP^C and aSyn. SPR was used to show that clathrin binds to recombinant PrP, but not aSyn. Experimental disruption of clathrin-coated vesicles significantly decreased aSyn internalization.
Conclusion: PrP^C's native trafficking can be misused to internalize misfolded aSyn through a clathrin-based mechanism, which may facilitate the spreading of pathological aSyn. Disruption of aSyn-PrP^C binding is, therefore, an appealing therapeutic target in α-synucleinopathies. © 2021 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: α-synuclein; α-synucleinopathies; cellular prion protein

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Tobias Thom and Matthias Schmitz contributed equally to this work.

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Lewy bodies (LB) are diagnostic hallmarks of certain α-synucleinopathies, including Parkinson's disease (PD) and dementia with Lewy bodies.[1-3] LBs are composed of α-synuclein (aSyn) aggregates, other proteins, lipids, and membranes.[4] aSyn has multiple physiological functions in synaptic biology.[2,5] aSyn can also misfold and accumulate, resulting in α-synucleinopathies, diseases characterized by subcortical multisystem degeneration with neuronal loss and gliosis.[6] Oligomerized aSyn (OL-aSyn) is cytotoxic in cell-based systems.[7] α-Synucleinopathies are thought to be caused by the internalization and accumulation of soluble OL-aSyn.[8,9]

The pathological propagation of α-synucleinopathies is consistent with a prion-like propagation. The pathogenic aSyn template induces the intracellular conversion of native aSyn into the pathogenic form, thereby amplifying the pathogenic conformation. The internalization of the pathogenic conformation facilitates its spread from neuron to neuron.[10-14] This internalization of extracellular aSyn has been proposed to occur by several different mechanisms.[15] OL-aSyn are internalized by endocytosis, implying a selective uptake mechanism.[16]

The cellular prion protein (PrP^C) is highly conserved and transiently resides on the surface of neurons and other tissues before it is internalized.[17] It has previously been shown to protect stressed cells.[18-20] PrP^C can be induced to refold into an infectious conformation (prion protein scrapie; PrP^{Sc}), which is internalized by a PrP^C-mediated mechanism to cause transmissible spongiform encephalopathies.[21,22] Previous studies posit that the native PrP^C internalization may also be misused to internalize other prion-like protein refolding diseases, such as amyloidopathies and α-synucleinopathies.[23-25] This hypothesis is supported by recent studies showing that PrP^C may facilitate the internalization of extracellular amyloid beta oligomers. Once internalized, these amyloid beta oligomers may be amplified intracellularly and influence disease progression by contributing to different cellular dysfunctions.[26-28] A similar mechanism of internalization has also been proposed for intercellular transfer of misfolded aSyn.[25] We recently demonstrated that OL-aSyn-induced long-term potentiation (LTP) hippocampal deficits[25] are mediated by PrP^C, suggesting it has a role in α-synucleinopathies.[29]

We bred four different lines of mice, each with or without *Prnp* ablated and exhibiting a rapid or slowly developing aSyn pathology. We developed two transgenic cell models, one that natively expresses low levels of PrP^C and the other that overexpresses PrP^C. These models were used to elucidate PrP^C's role in α-synucleinopathies. In addition, we used mass spectrometry to define the interactome of PrP^C and aSyn. Surface plasmon resonance (SPR) was used to determine the binding constants of PrP^C with monomeric-aSyn (mono-aSyn) and OL-aSyn.

Material and Methods

Behavioral Testing of Mice

Behavioral tests (Supplemental Material S1) were performed according to previously described protocols.[30] To avoid potential influence of sex, we used male mice in our behavior studies.

Cell Culture

Human neuroblastoma cell line SH-SY5Y (SHWT) was maintained in Dulbecco's modified Eagle medium (Biochrom, Berlin, Germany) containing 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine at 37°C, 5% CO_2, and 95% humidity. A previously described transfection procedure was used to generate a cell line that permanently overexpressed PrP^C.[31]

Preparation of Primary Cortical Neurons

Cells were prepared with some modification according to a previously published protocol (Supplemental Material S1).[20,32]

Cell Treatment with aSyn

SHWT cells were plated onto six-well plates to approximately 90% confluence. The treatment occurred according to the following protocol (Supplemental Material S1).

Fractionation

Cells and mouse brain homogenates were partitioned into cytosolic, membrane, nuclear, and cytoskeletal fractions using the Qproteome Cell Compartment Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Supplemental Material S1).

Western Blot Analyses

Western blot was performed using previously described protocols (Supplemental Material S1).[30,33]

Colocalization Studies by Confocal Laser Microscopy

Colocalization studies used fluorescent dye-labeled antibodies and nucleic acid staining dyes to determine the locations of PrP^C, aSyn, and the nucleus. Anti-PrP^C mouse monoclonal (PrP specific antibody, scrapie associated fibrils, SAF32) and anti-aSyn rabbit monoclonal (MJFR1) antibodies (0.5 μg/mL) facilitated colocalization analysis (Supplemental Material S1).

Determination of Total-aSyn and OL-aSyn by Enzyme-Linked Immunosorbent Assay

The measurement of total-aSyn and OL-aSyn was determined by enzyme-linked immunosorbent

assay (ELISA; Euroimmun/ADx, Gent, Belgium) following the manufacturer's instructions (Supplemental Material S1).

Co-Immunoprecipitation

The monoclonal anti-aSyn (MJFR1; Abcam, Cambridge, UK) and anti-PrP^C (SAF32; Bertin Bioreagent, Hamburg, Germany) antibodies were bound to magnetic Dynabeads according to the manufacturer's instructions (Supplemental Material S1).

Production of Recombinant Human PrP^C

Full-length recombinant human PrP^C (rHuPrP) expression was induced by the Overnight Express Autoinduction System 1 (Catalog No. 71300-4; Novagen, Madison, WI), according to the manufacturer's instructions (Supplemental Material S1).

Statistical Analysis

All data analytic results are representative of three independent experiments. Data were evaluated using GraphPad Prism 6.01 statistical software (GraphPad Software, San Diego, CA; Supplemental Material S1).

1.12 Ethics Approval

The study has been approved by the Lower Saxony State Office for Consumer Protection and Food Safety (No. 16/2073).

Results

PrP^C Knockout Partly Rescues Deficits in ThySyn and TgM83 Mice Accompanied with Higher OL-aSyn Levels and a Changed Cellular Localization of aSyn

We created two transgenic mouse lines to study PrP^C's role in α-synucleinopathies. The first line (ThySynPrP00) was a cross between a more rapidly progressive α-synucleinopathy phenotype (ThySyn)[34] and a *Prnp* ablated (PrP00) mouse line (Zurich 1).[35] The second mouse line (TgMPrP00) was a cross between a line exhibiting a familial PD mutation (TgM83) displaying a mild-progressive phenotype with PrP00 mice. In addition, we used a wild-type (WT) mouse line (C57BL/6J) and a PrP00 (C57BL/6J) mouse line. These animals and their tissues were used in our experiments to assess the effect of ablating PrP^C expression on aSyn-associated phenotypes (Supplemental Material S1).

To determine if *Prnp* ablation altered native aSyn expression, different forms of aSyn were measured in the brains of our transgenic mice. ELISA-based measurement showed no significant differences in total-aSyn levels. However, OL-aSyn levels were significantly lower in ThySynPrP00 compared with ThySyn mice (Fig. 1 A1,A2) and in TgMPrP00 compared with TgM83 mice (Fig. S1A1,A2). Three phosphorylated analogs of aSyn (p-aSyn) (phosphorylation at serine 87, tyrosine 125, and serine 129 [p87, p125, and p129]) were assayed by Western blots of the cerebral cortex from the four transgenic mouse lines (ThySyn, ThySynPrP00, TgM83, TgMPrP00). In 9-month-old ThySyn and ThySynPrP00 mice, the levels of the three different phosphorylated forms of aSyn were not significantly different (Fig. 1B). Analogous experiments were performed on TgM83 and TgMPrP00 mice and comparable results (Fig. S1B) were observed, suggesting that ablating *Prnp* does not influence the endogenous aSyn or p-aSyn levels.

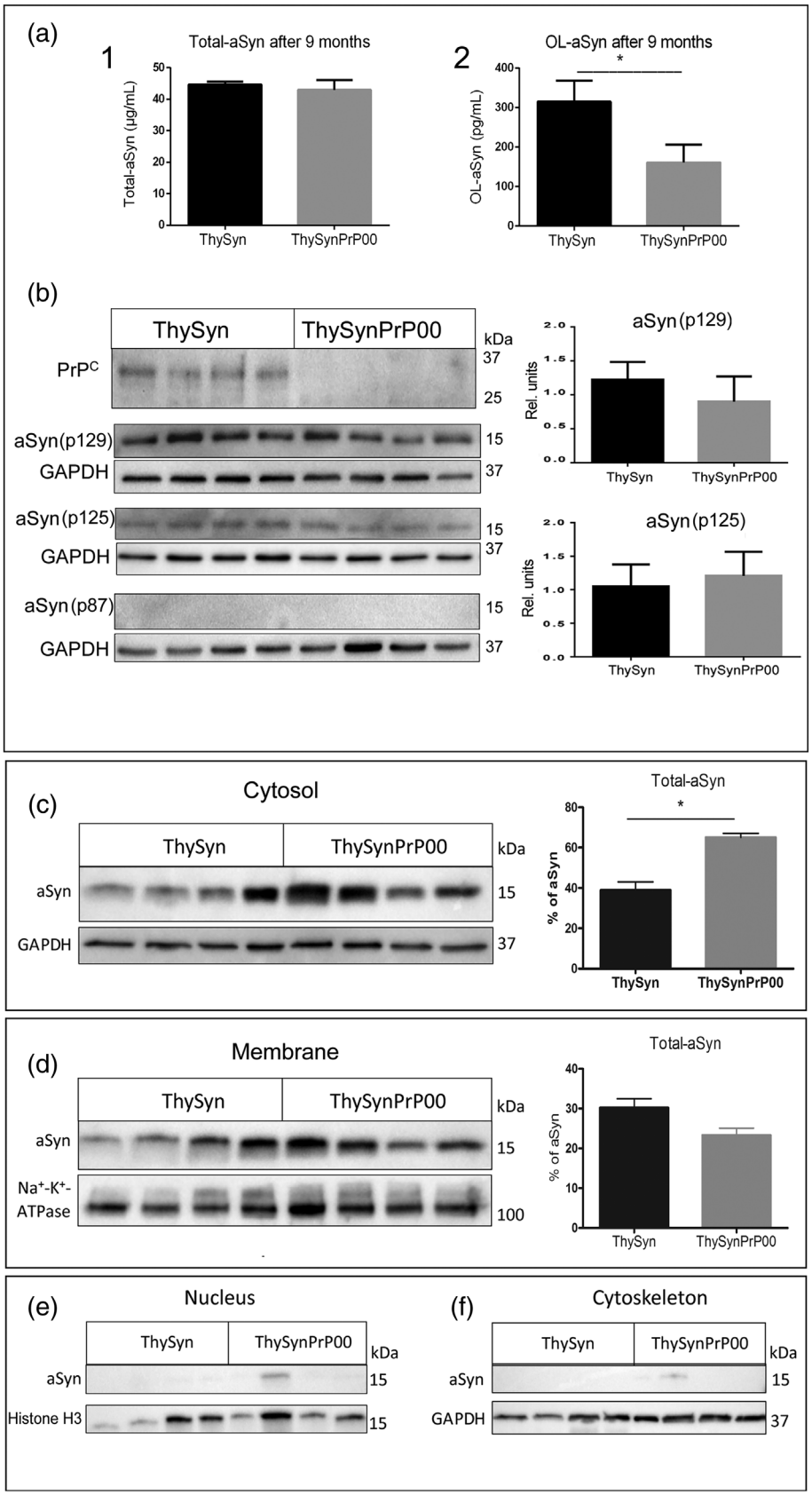
We wanted to determine the subcellular locations of PrP^C and aSyn in the cerebral cortex. Samples were partitioned into four cellular fractions (cytosol, membrane, cell nucleus, and cytoskeleton). Housekeeping proteins (glyceraldehyde-3-phosphate-dehydrogenase [GAPDH], histone 3, and Na^+-K^+-adenosine triphosphatase (ATPase)) were used as loading controls for the different cellular fractions (Fig. 1C–F). aSyn levels were higher in the cytosolic fraction of ThySynPrP00 mice compared with ThySyn mice (Fig. 1C); in the membrane fraction, they tended to be higher without significance (Fig. 1D). The total expression levels and the subcellular localization of PrP^C were not significantly changed (only a tendency of lower PrP^C levels in the membrane) in ThySyn and TgM83 mice compared with WT mice (Fig. S2A–F). A subcellular colocalization with aSyn could be observed in the cytosol (Figs. 1C–F and S2B-F).

Ablation of PrP^C Extends the Life Span of aSyn Transgenic Mice

The lifespans of ThySynPrP00 versus ThySyn mice and TgMPrP00 versus TgM83 mice were determined. Others have shown that the lifespan of PrP00 and WT mice are comparable, indicating that *Prnp* ablation does not affect a mouse's lifespan.[35] We observed that the lifespan of the ThySynPrP00 mouse line was significantly longer ($P < 0.01$) than that of the ThySyn line (Fig. 2A). Analogously, the lifespan of the *Prnp*-ablated TgMPrP00 ($n = 33$) mouse line was significantly longer ($P < 0.05$) than that of the PrP^C-expressing TgM83 ($n = 31$) mouse line (Fig. S1C). Together, these data indicate that ablation of *Prnp* can moderate aSyn-associated reductions in lifespan.

Prnp Knockout Reduces Behavioral Deficits Related to Misfolded aSyn In Vivo

We monitored *Prnp* ablation's role in moderating behavioral deficits, induced by an aSyn pathology, by



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comparing ThySyn with WT mice and ThySynPrP00 with PrP00 mice. We previously showed that older (9–24 months) PrP00 mice show behavioral changes.[30,36] We used six behavioral tests to analyze the influence of *Prnp* ablation in transgenic mice: nest building (adaptive behavior), open field (locomotor activity), novel object recognition (memory), elevated plus maze (EPM; anxiety), fear conditioning (associative learning), and rotarod (motoric skills). The first comparison (ThySyn with WT) revealed significant differences in locomotor behavior, associative learning, cognition, and anxiety levels after 9 months. Interestingly, ablation of *Prnp* in ThySynPrP00 mice partly rescued the behavioral abnormalities in locomotor activity, anxiety, associative learning (context and tone dependent), and memory performance, which occurred in ThySyn mice (Fig. 2B–F). We did not observe deficits in the rotarod task with either ThySyn or WT mice. The observed deficits in nesting and locomotor activity in the ThySyn, but not WT, mice suggests moderate motoric dysfunctions in ThySyn mice (Fig. 2B,H,I).

A second mouse line, TgM83, exhibits a moderate aSyn pathology phenotype that permitted the investigation of behavioral differences at older ages. Behaviors were compared between TgM83 and WT mice and between TgMPrP00 and PrP00 mice at ages 9 and 18 months. TgM83 mice demonstrated greater basal anxiety levels than the WT mice, starting at 9 months and increasing by 18 months (Fig. S1D). No significant difference in basal anxiety levels could be detected between TgMPrP00 and PrP00 mice at the ages. In addition, nest-building deficits in TgM83 compared with WT mice were observed in 18-month-old mice (Fig. S1E), whereas the rotarod test performance remained unchanged (Fig. S1F).

PrP^C Promotes aSyn Internalization In Vitro

We used the human neuronal cell lines, SH-SY5Y with PrPC over expression (SHPrP) and SH-SY5Y (SHWT) to study the influence of PrP^C levels on the uptake of aSyn. The SHWT cell line natively expresses extreme low levels of PrP^C. The SHPrP line was engineered to overexpress levels of PrP^C (approximately fourfold) than the SHWT line (Fig. S3A,B). Each cell line was incubated for 12 hours with a nontoxic concentration (1 μM) of either added recombinant mono-aSyn or OL-aSyn (Fig. 3A2,B2). Incubation with 1 μM of mono-aSyn was sufficient to detect a significant difference in uptake (without toxicity).[37] Transmission electron microscopy (TEM) analysis of mono-aSyn and OL-aSyn showed that the monomeric solution contained no aSyn aggregates or fibrils, whereas the aggregated aSyn fraction contained a mixture of oligomeric and fibrillary aSyn species (Fig. 3A2,B2). Western blot signals of internalized mono-aSyn and OL-aSyn were quantified by densitometry. Our data indicated that SHPrP cells incubated with mono-aSyn had a significantly higher increase (≈1.5-fold) in levels of intracellular mono-aSyn compared with similarly treated SHWT cells (Fig. 3A1–3). Cells treated with OL-aSyn showed higher amounts of internalized OL-aSyn (an approximately fourfold change) in SHPrP than in SHWT cells (Fig. 3B1-3). These results are consistent with a PrP^C-dependent internalization of OL-aSyn.

Subsequently, we quantified the amount of phosphorylated-aSyn (p-129aSyn [p129]) after both cell lines (SHPrP, SHWT) were treated either with mono-aSyn or OL-aSyn. Treatment of SHPrP or SHWT cells with mono-aSyn yielded nonphosphorylated internalized aSyn (Fig. S4A, B). Treating SHWT cells with OL-aSyn yielded a similar result. However, when SHPrP cells were treated with OL-aSyn, a significant increase of internalized p129 aSyn was observed (Fig. S4A,B). These results are consistent with a PrP^C-dependent internalization of OL-aSyn followed by phosphorylation by endogenous kinases.

We performed a series of aSyn (human) uptake experiments using cortical neurons derived from WT and PrP00 mice. TEM analysis of the OL-aSyn or mono-aSyn fraction showed that the OL-aSyn also contained soluble aSyn fibrils, whereas the mono-aSyn did not (Fig. S5A2,B2). Primary cortical neuronal cells were isolated and then cultivated for 7 days to allow process formation. They were incubated with nontoxic amounts (1 μM) of mono-aSyn and OL-aSyn. Western blot data were quantified by densitometry, which revealed that WT-derived cells incorporate significantly

FIG. 1. Detection of different forms of aSyn and their subcellular distribution in ThySyn and ThySynPrP00 mice. Enzyme-linked immunosorbent assay measurement showing equal total-aSyn levels and a higher amount of soluble recombinant aSyn fraction consisting of oligomers and small fibrils (OL-aSyn) in ThySyn compared with ThySynPrP00 mice (**A1, A2**). Western blots showing p-aSyn (p125 and p129) levels and a graphical summary of those data. aSyn and p-aSyn (p125 and p129) levels are similar in 9-month-old ThySyn and ThySynPrP00 mice (**B**). Brain homogenates were separated into four fractions (cytosol, membrane, cell nucleus, and cytoskeleton). In ThySynPrP00 mice, aSyn levels were detected by Western blot. Percentages of aSyn levels in each fraction were calculated in relation to total amounts of aSyn in all fractions (only indicated for cytosol and membrane). ThySynPrP00 mice indicated a higher amount of aSyn in the cytosolic fraction compared with ThySyn mice. GAPDH, histone 3, and Na^+-K^+-adenosine triphosphatase (ATPase) represent specific loading controls for different cellular compartments (**C–F**). Data are presented as mean ± standard deviation (n = 8 per experimental group). The two groups were compared using the Wilcoxon Mann–Whitney test. The number of asterisks indicates the significance level: $^*P < 0.05$. aSyn, α-synuclein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OL-aSyn, oligomeric-aSyn; p-aSyn, phosphorylated analogs of aSyn.

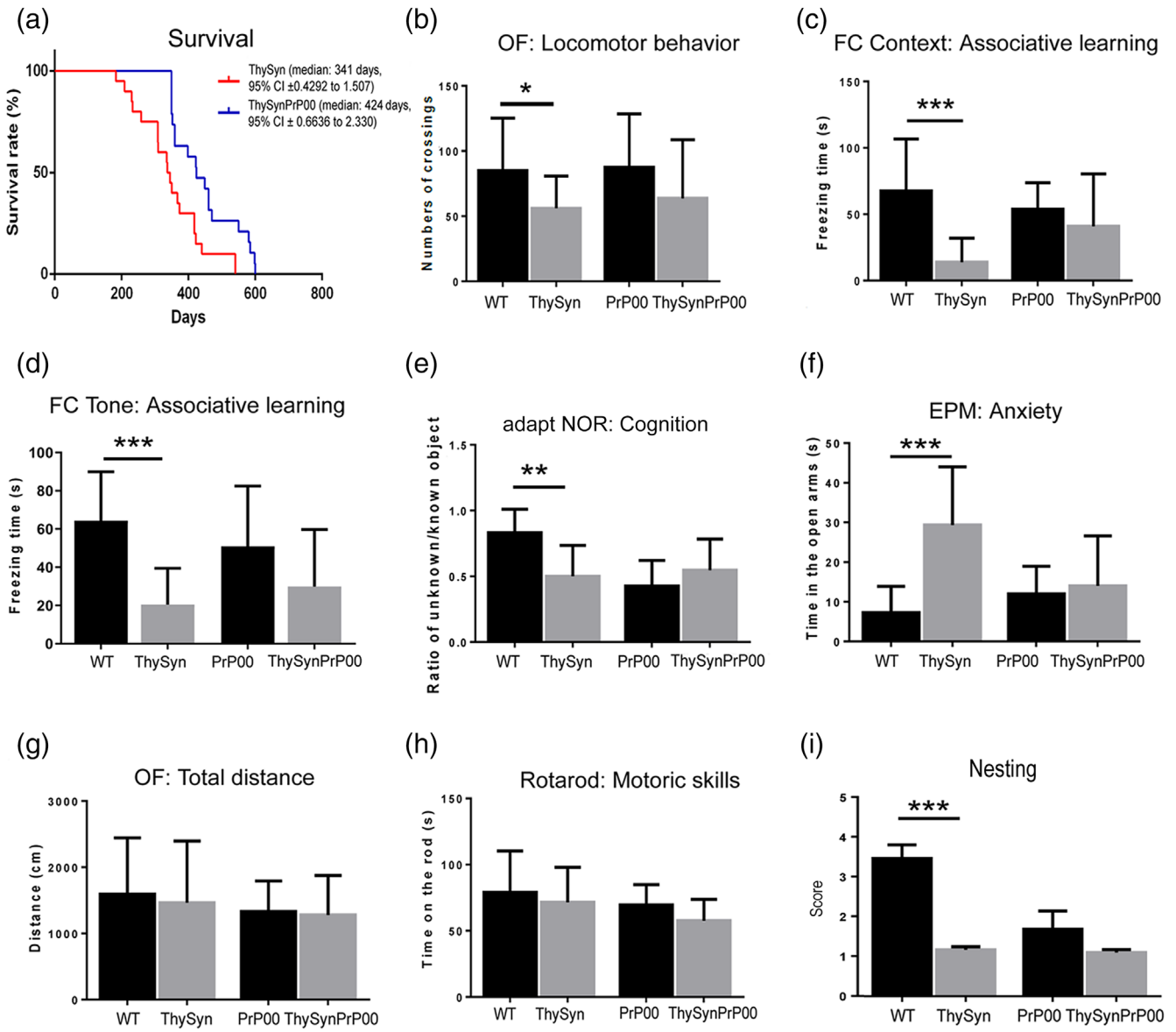
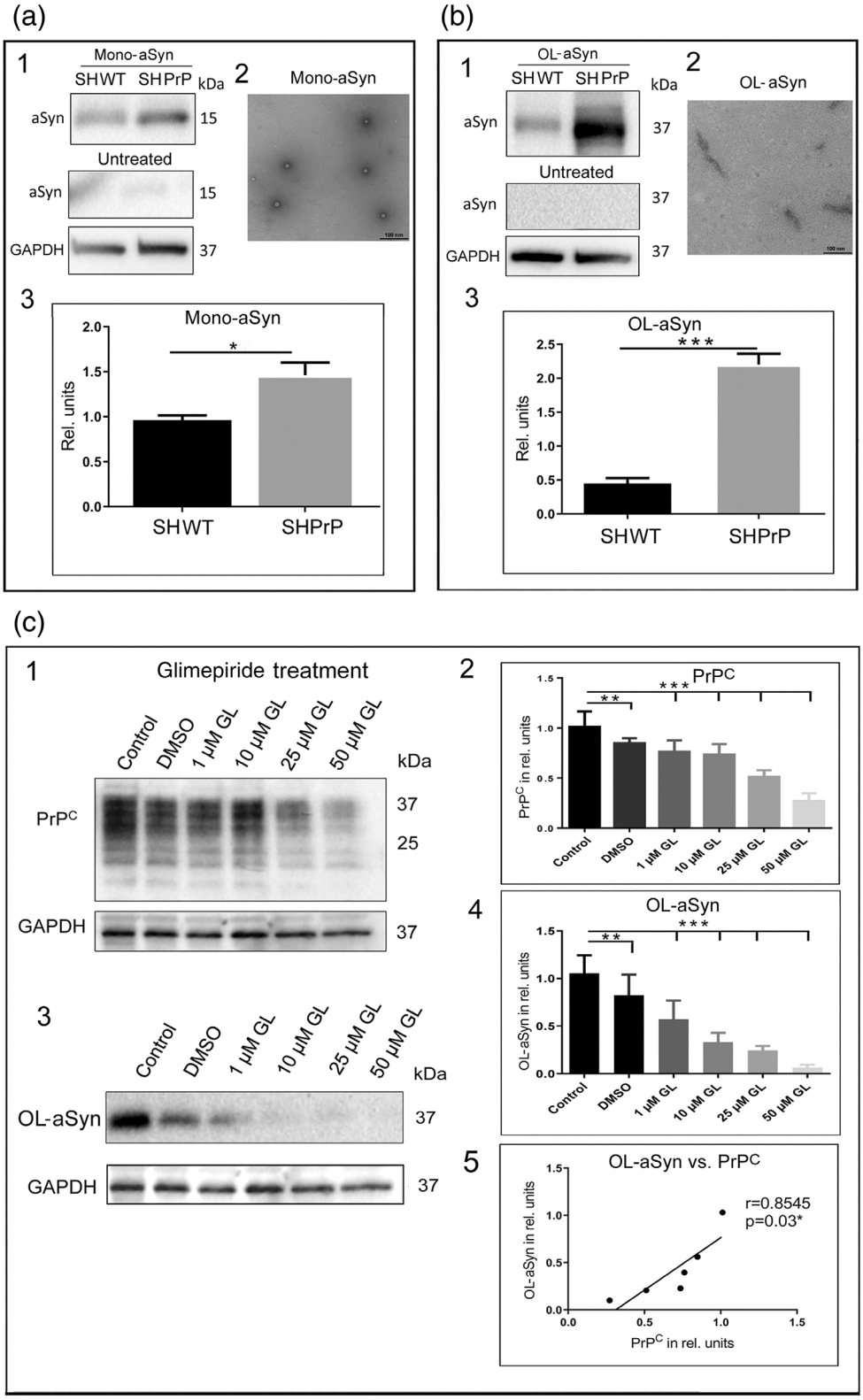


FIG. 2. PrP^C facilitates aSyn-induced toxicity as indicated by decreased survival times and observed behavioral deficits at 9 months. The lifespan of ThySyn mice (n = 28) is significantly reduced (341 days; 95% CI ± 0.4292 to 1.507) compared with ThySynPrP00 (n = 28; 424 days; 95% CI ± 0.6636 to 2.330) mice ($P < 0.01$; Mantel Cox test, Gehan-Breslow-Wilcoxon test) (**A**). Locomotor activity (measured by grid crossings in the open field test) was decreased in ThySyn mice compared with WT mice, but not in ThySynPrP00 compared with PrP00 mice (**B**). Context-dependent and tone-dependent associative learning (measured by the fear conditioning test) was decreased in ThySyn mice compared with WT mice, but not in ThySynPrP00 and PrP00 mice (**C**,**D**). Cognitive performance and curiosity were measured by the novel object recognition test. Mean duration of exploration of the novel object in relation to the familiar one was reduced in ThySyn mice compared with WT mice but not in ThySynPrP00 mice compared with PrP00 mice (**E**). ThySyn mice exhibited a decline in basal anxiety compared with WT mice, as shown by the significantly greater time spent in open arms in the EPM. ThySynPrP00 mice showed no significant differences compared with PrP00 mice (**F**). No significant differences could be detected within the two sets of mouse lines in terms of the total distance traveled in the open field or the rotarod test (time spent on the rod) (**G**,**H**). Nesting was significantly decreased in ThySyn mice compared with WT mice after 9 months (**I**). The two groups (WT vs. ThySyn and PrP00 vs. ThySynPrP00) were compared using the Wilcoxon Mann–Whitney test. The number of asterisks indicates the significance level: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Values are depicted as mean ± standard deviation. N = 12 per experimental group. aSyn, α-synuclein; CI, confidence interval; EPM, elevated plus maze; FC, fear conditioning; NOR, novel object recognition; OF, open filed; PrP^C, cellular prion protein; PrP00, Prnp knockout; WT, wild-type. [Color figure can be viewed at wileyonlinelibrary.com]

more mono-aSyn and OL-aSyn by an approximately fourfold or 60-fold change compared with the Prnp00-derived cells (Fig. S5A1,A3,B1,B3). Again, these results are consistent with a PrP^C-dependent internalization of aSyn.

Glimepiride treatment induces the cleavage of the protein portion of PrP^C from the surface of cells, such as SHPrP cells that overexpress it.[38] Before aSyn treatment, SHPrP cells were incubated with varying concentrations (0–50 μM) of glimepiride for 2 hours. After incubation,

cellular proteins were analyzed by Western blot. The intensities of the PrP^C bands were quantified by densitometry (Fig. 3C1–2). These data indicate that reducing the amount of natively expressed PrP^C on a cell's surface results in a lower amount of internalized aSyn (Fig. 3C1–4). Based on a Pearson correlation analysis, the amounts of



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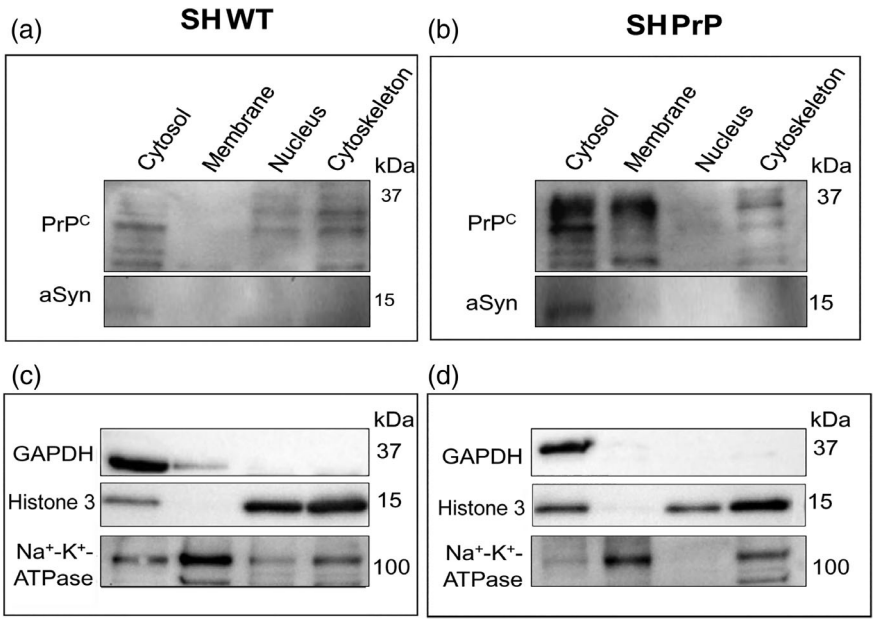

FIG. 4. Cellular localization of PrP^C and aSyn. Detection of PrP^C and aSyn in one of the four subcellular fractions (cytosol, membrane, cell nucleus, and cytoskeleton) from SHWT and SHPrP cells after a 12-hour treatment with aSyn (1 μM). In SHPrP cells, aSyn and PrP^C were mainly colocalized in the cytosol fraction that is barely visible in SHWT cells (**A**, **B**). All protein bands between 37 kDa and 25 kDa represent different glycoforms or truncated forms of PrP^C. The main glycoforms are di-, mono- and un-glycosylated PrP^C between 29 and 37 kDa (**A–D**). GAPDH, histone 3, and Na^+-K^+-ATPase represent specific loading controls for different cellular compartments. aSyn, α-synuclein; ATPase, adenosine triphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PrP^C, cellular prion protein; SHPrP, SH-SY5Y with PrPC over expression; SHWT, SH-SY5Y wild-type PrPC.

expressed PrP^C and intracellular aSyn were correlated (Fig. 3C5). This suggests that (glycosylphosphatidylinositol [GPI] anchor-bound) PrP^C is required for the internalization of extracellular aSyn.

PrP^C and aSyn Colocalize in the Cytosol In Vitro

Four subcellular fractions of SHPrP and SHWT cells were isolated to determine the extent of PrP^C and aSyn colocation in each fraction. After incubating SHPrP and SHWT cells with mono-aSyn for 12 hours, the cells were washed, homogenized, and separated into four distinct fractions. Each fraction was analyzed by Western blot. PrP^C is found in comparable amounts in both the membrane and cytosol fractions. Both PrP^C and aSyn were found colocalized in the cytosolic fractions of SHWT and SHPrP cells. Higher levels of both PrP^C and internalized aSyn were found in SHPrP than in SHWT cells (Fig. 4A–D).

Colocalization and Interaction of PrP^C With aSyn in SHPrP Cells

The locations of PrP^C and aSyn were visualized by immunocytochemistry. The images (green) show, as expected, that SHPrP cells expressed higher levels of PrP^C than SHWT cells (Fig. S6A,B). The images (total-aSyn in red) display higher signal intensities when incubated with OL-aSyn, particularly in SHPrP cells. The overlap of the aSyn and PrP^C signals is a measure of colocalization that was quantified by calculating the Manders' overlap coefficient. Based on this analysis, SHPrP cells treated with OL-aSyn showed a significantly higher colocalization coefficient compared with SHPrP treated with mono-aSyn or SHWT cells treated with mono-aSyn or OL-Syn (Fig. S6C).

FIG. 3. Cellular internalization of aSyn is facilitated by PrP^C. SHWT and SHPrP cells were treated for 12 hours with recombinant mono-aSyn and OL-aSyn (1 μM). After 12 hours, the levels of internalized mono-aSyn (15 kDa) and OL-aSyn (37 kDa) were readily detected by Western blot in treated cells, whereas untreated cells showed barely detectable amounts of endogenous aSyn (**A1**, **B1**). Transmission electron microscope images of recombinant mono-aSyn and OL-aSyn solutions show the absence and presence of fibrils, respectively (**A2**, **B2**). Graphs of the densitometric quantification aSyn uptake in SHWT and SHPrP cells (**A3**, **B3**). SHPrP cells were treated with glimepiride (GL) (1–50 μM) for 2 hours and then incubated for 12 hours with OL-aSyn. GL treatment results in the cleavage of PrP^C's GPI anchor, releasing it from the plasma membrane and into the medium. With increasing concentrations of GL, a smaller amount of membrane-bound PrP^C was detected (**C1**, **C2**). Densitometric quantification showed a gradual dose-dependent decrease of internalized OL-aSyn as the concentration of GL is increased (accompanied by a decrease in PrP^C) (**C3**, **C4**). A P value <0.001 is considered as extremely significant (***), <0.01 very significant (**), <0.05 significant (*), and ≥0.05 not significant (ns). aSyn, α-synuclein; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GL, glimepiride; GPI, glycosylphosphatidylinositol; mono-aSyn, monomeric-aSyn; OL-aSyn, oligomeric-aSyn; PrP^C, cellular prion protein; SHPrP, SH-SY5Y with PrPC over expression; SHWT, SH-SY5Y wild-type PrPC; WT, wild-type.

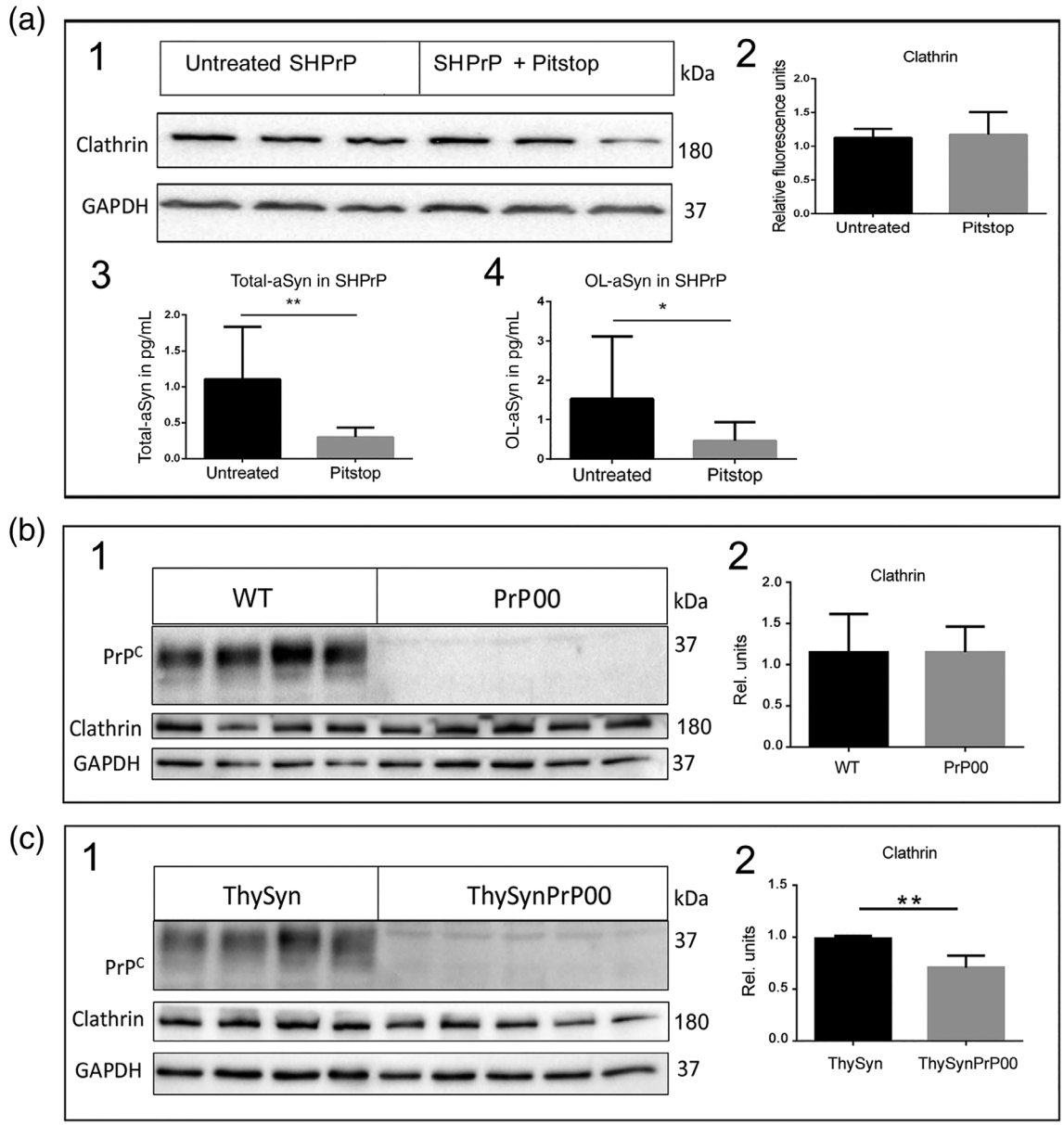

FIG. 5. Clathrin is a mediator of PrP^C-facilitated internalization of extracellular aSyn. When SH-SY5Y with PrP^C overexpression (SHPrP) cells were treated with a clathrin inhibitor Pitstop 2 (Pitstop) the expressed levels of clathrin remained unchanged; enzyme-linked immunosorbent assay–based analysis showed that significantly less total-aSyn and OL-aSyn was internalized (**A**). Western blot analysis showed comparable amounts of expressed clathrin in WT and PrP00 mice, indicating that *Prnp* ablation does not perturb clathrin protein expression levels (**B**). ThySyn mice showed a significantly higher level of expressed clathrin compared with ThySynPrP00 mice (**C**). A *P* value <0.001 is considered as extremely significant (***), <0.01 very significant (**), <0.05 significant (*), and ≥0.05 not significant (ns). aSyn, α-synuclein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OL-aSyn, oligomeric-aSyn; PrP^C, cellular prion protein; SHPrP, SH-SY5Y with PrPC over expression; SHWT, SH-SY5Y wild-type PrPC; WT, wild-type.

Common Interaction Partners of PrP^C and aSyn

Cortical brain homogenates from ThySyn mice were immunoprecipitated using Dynabeads coupled with either a PrP^C-specific (SAF32 [mouse]) or an aSyn-specific (MJFR1 [rabbit]) monoclonal antibody. Dynabeads incubated with nonspecific mouse or rabbit immunoglobulin G showed no signals (data not shown). Western blot–based analysis showed the presence of both PrP^C and aSyn in the brain homogenate prior to immunoprecipitation (Fig. S7A1–3). PrP^C (29–36 kDa) and aSyn (15 kDa) were present in both immunoprecipitates (Fig. S7A1–3), confirming an in vivo PrP^C–aSyn interaction.

Co-immunoprecipitates (co-IPs) were analyzed via qualitative mass spectrometry to define the interactome of PrP^C and aSyn. This allows a precise characterization of PrP^C and aSyn-binding partners with the goal of identifying other proteins that may be involved in PrP^C-mediated aSyn uptake. A total of 131 proteins were identified in the PrP^C co-IP. For the aSyn co-IP,

42 proteins were identified by mass spectrometry. Of these 173 proteins, 38 were common to both co-IPs (Fig. S7B; Tables S1–S3). The Uniprot (https://www.uniprot.org/) database was used to identify their known or purported physiological function. Most are involved in protein binding and catalytic processes (Fig. S7C). Of the 38 common interaction partners, we noticed that clathrin had the highest spectral count value (Tables S1–S3), suggesting that it might be involved in the PrP^C-mediated internalization of aSyn. Clathrin forms the triskelia that create cage-like lattices that facilitate classical endocytosis via clathrin-coated pits.[39]

Inactivation of Clathrin-Coated Vesicles Reduces the Uptake of aSyn via PrP^C

The drug Pitstop 2 disrupts the formation of clathrin-coated vesicles.[40] We used it to evaluate clathrin's role in the PrP^C-mediated uptake of aSyn. Treatment of SHPrP cells with Pitstop 2 did not change the levels of expressed clathrin. SHPrP cells were treated with Pitstop 2 and incubated with OL-aSyn. ELISA-based analysis of intracellular (total and OL) aSyn revealed that Pitstop 2–treated cells contained less intracellular aSyn compared with untreated SHPrP cells (Fig. 5A1-4). Western blot was used to quantify the expressed levels of clathrin in ThySyn versus ThySynPrP00 mice and WT versus PrP00 mice. ThySynPrP00 mice showed significantly less clathrin protein than ThySyn mice. In contrast, WT and PrP00 mice expressed similar levels of clathrin, suggesting clathrin partners in the PrP^C-mediated internalization of aSyn (Fig. 5B1–2 and C1-2).

SPR Studies of rHuPrP, OL-aSyn, and Clathrin Binding

SPR was used to measure the binding of mono-aSyn and OL-aSyn to recombinant human PrP^C (rHuPrP). Interestingly, we found a direct protein–protein interaction between rHuPrP (ligand) and aSyn (analyte; mono and OL; Fig. S8A,B). In contrast, recombinant chimeric-hamster-sheep PrP (negative control) failed to bind aSyn (Fig. S8C). Anti-PrP^C mAb-12F10 was used as a positive control (Fig. S8D). In addition, we observed a direct interaction between rHuPrP and clathrin, but not between clathrin and OL-aSyn (Fig. S8E, F). SPR data were used to calculate the binding constants for rHuPrP to mono-aSyn, OL-aSyn, or clathrin. The 1:1 Langmuir model was applied to mono-aSyn binding[41] and the bivalent analyte model for OL-aSyn binding because OL-aSyn may exhibit more than one binding site. The equilibrium constant (K_D) value of mono-aSyn ($K_D = 3.70E\text{-}09$) was higher than the K_D values of OL-aSyn ($K_D = 2.39E\text{-}09$), indicating a higher binding affinity of rHuPrP to OL-aSyn (Fig. S8A, B). The SPR-based K_D for the rHuPrP–clathrin interaction was found to be 2.34E-10 (Fig. S8E).

Discussion

Various experimental models have shown that PrP^C has a role in internalizing the β-sheet rich forms of amyloid beta and tau, thereby facilitating their spread among cells.[29,42-44] This implies an interaction and a colocalization of PrP^C with those misfolded amyloid proteins.[26,45-47] Researchers showed the important role PrP^C has in aSyn internalization.[29] Ablating *Prnp* in cortical neurons rescues them from OL-aSyn-induced inhibition of LTP.[25] Others reported that OL-aSyn does not bind PrP^C nor does it have a role in α-synucleinopathies.[48] The evidence for PrP^C's role in internalizing aSyn is, therefore, contradictory and needs to be resolved.

To clarify the role of PrP^C in facilitating the cell-to-cell spread of α-synucleinopathies, we created two *Prnp*-ablated transgenic mouse lines with different aSyn pathologies. The first line displayed a more rapidly progressive phenotype (ThySyn).[34,35] The second mouse line displayed a milder phenotype and contained a familial PD mutation (TgM83). Both *Prnp*-ablated lines exhibited an increase in lifespan compared with the PrP^C-expressing progenitor ThySyn or TgM83 lines. After 9 months, behavioral deficits in locomotor activity, nest building, memory function, and associative learning and a decrease in basal anxiety were observed in ThySyn mice, but not in the ThySynPrP00 mice. In TgMPrP00 mice, the rescue of aSyn-induced behavioral deficits was partially confirmed. aSyn-related motoric deficits, not detected by the rotarod test, were confirmed by other behavioral tests such as nesting and open field performance. These inconsistent results may be a consequence of different test protocols and more sensitive motor tests such as wire hanging, nesting, and gnawing being used.[49] The total expressed levels of aSyn and p-aSyn were not significantly different in ThySyn versus ThySynPrP00 or TgM83 versus TgMPrP00 mice. We observed a significant decrease in OL-aSyn as well as a change in the subcellular localization of aSyn after PrP knockout in mice expressing mutated aSyn. These observations support the hypothesis that PrP^C facilitates the internalization of misfolded aSyn, resulting in aSyn-mediated toxicity. Furthermore, the PrP^C-mediated internalization of extracellular misfolded aSyn is an important mechanism for the spread of α-synucleinopathies among cells.[11,12,50,51]

We showed that PrP^C preferentially favors internalizing OL-aSyn (an approximately fourfold change) over mono-aSyn (≈1.5-fold change). Our results confirm those of other researchers who demonstrated that PrP^C internalizes soluble misfolded aSyn. *Prnp* ablation has been shown to partly rescue cells from the functional impairment of LTP.[29] This implies an important role for PrP^C in the internalization of aSyn required for intercellular spread of α-synucleinopathies.

Treatment of PrP^C-expressing cells with glimepiride cleaves the protein portion of PrP^C from the cell surface. We observed a dose-dependent decrease in aSyn levels after treating SHPrP cells with glimepiride. This supports the hypothesis that PrP^C's native membrane trafficking is important for aSyn internalization. These results were further confirmed by the PrP^C-mediated uptake of OL-aSyn in primary cortical neurons from WT mice when compared with analogous cells from PrP00 mice. These cell-based results show the dependence of aSyn internalization on the presence and expression levels of PrP^C.

Cellular fractionation and immunocytochemical costaining showed that aSyn and PrP^C are colocalized. In PrP^C-overexpressing (SHPrP) cells, comparable amounts of PrP^C are found in the membrane and cytosol fractions, while subtantial amounts of aSyn were detected in the cytosol of these cells. These data support the hypothesis that aSyn internalization is facilitated by PrP^C. We used the ThySyn mouse line and a co-IP to demonstrate an interaction between aSyn and PrP^C in vivo, which was consistent with previous studies.[25,29]

Our work also demonstrated that, after incubation with either mono-aSyn or OL-aSyn, PrP^C colocalized with internalized aSyn. Previous work, using several anti-PrP^C antibodies raised against different epitopes on PrP^C, showed that amino acids 93 to 109 of PrP^C are involved in the binding with aSyn.[25,29]

Our SPR experiments employed recombinant human PrP^C to show direct PrP-aSyn binding. Other researchers isolated native murine PrP^C for their SPR-based aSyn-binding studies. They were unable to show binding, a crucial element for the receptor hypothesis.[48] This lack of binding may be due to the improper folding of the glycosylated and GPI anchor-containing PrP^C protein, use of an inappropriate capture antibody, use of aSyn from brain homogenates, and other differences in experimental protocol. By using immobilized purified recombinant prion protein (rPrP) (the same secondary and tertiary structure as PrP^C; no GPI anchor or glycosylation), we showed a direct interaction of rPrP with both forms of aSyn. The equilibrium dissociation constants (K_D) of the rPrP/mono-aSyn and or rPrP/OL-aSyn complex were determined to be 3.70E-09 and 2.39E-09, respectively, indicating a higher binding affinity of PrP^C to OL-aSyn.

Previously, we proposed that aSyn and PrP^C may form a complex that induces metabotropic glutamate receptor 5 to phosphorylate Fyn kinase.[25] This interaction suggested a possible link to clathrin. We used qualitative mass spectrometry to determine the interactome of the co-IPs of aSyn and PrP^C. Clathrin was the protein with the highest number of spectral counts that was common to both co-IPs. Interestingly, clathrin forms triskelia that build clathrin-coated vesicles that facilitate endocytosis.[52] For this reason, it was chosen for further analysis.

Our other SPR results showed a direct interaction between PrP^C and clathrin. Because the SPR data related to aSyn and clathrin binding do not correspond to co-IP findings, we conclude that aSyn may be indirectly attached to clathrin via PrP^C. Our other data indicate that levels of clathrin protein are significantly lower in ThyPrP00 compared with ThySyn mice. Expressed clathrin levels are comparable in WT and PrP00 mice, so simple *Prnp* ablation is not responsible for this observed reduction. Other researchers showed that PrP^C is initially localized in lipid rafts. When PrP^C binds copper, its conformation is distorted, which allows copper-bound PrP^C to migrate from the lipid raft to the detergent soluble portion of the plasma membrane, where it can be endocytosed via clathrin-coated pits.[53] We showed that Pitstop 2–treated SHPrP cells show a significant dose-dependent reduction of aSyn internalization. Because Pitstop 2 is an inhibitor of vesicle formation, this implies that clathrin-mediated endocytosis is involved in aSyn internalization. Other work on mesenchymal stem cells showed a protective effect of blocking clathrin-mediated endocytosis of extracellular aSyn, which aligns with our findings.[54] We suggest that binding of OL-aSyn to PrP^C induces a conformational change that permits this complex to migrate from the lipid raft region into detergent soluble regions of the cell membrane. Once in the detergent soluble region, OL-aSyn-bound PrP^C can be internalized by clathrin-coated vesicles (Fig. S9).

This work provides evidence for one possible uptake mechanism. Alternative mechanisms to internalize misfolded aSyn are possible, including tunneling nanotubes or exosome release.[55,56] In the future we plan to confirm the PrP^C-clathrin-mediated internalization using in vivo models and translational studies. We also plan to quantify the relative contribution of PrP^C-mediated aSyn internalization and intercellular spread on disease progression.

In conclusion, our data provide in vivo and in vitro evidence supporting a molecular mechanism for the PrP^C-mediated internalization of aSyn. This mechanism is a plausible explanation for the observed OL-aSyn-induced phenotypes in two different mouse models. Furthermore, our work highlights potential therapeutic targets (PrP^C, clathrin) to impede aSyn internalization. A disruption could prevent the intercellular movement of OL-aSyn and, therefore, potentially prevent or slow the progression of α-synucleinopathies. ■

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Data

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