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Abstract

Recent evidences suggest that disease progression in Parkinson's disease (PD) could occur by the spreading of α-synuclein (α-syn) aggregates between neurons. Here we studied the role of astrocytes on the intercellular transfer and fate of α-syn fibrils, using *in vitro* and *ex vivo* models. α-Syn fibrils can be transferred to neighboring cells, however the transfer efficiency changes depending on the cellular types. We found that α-syn is efficiently transferred from astrocytes-to-astrocytes and from neurons-to-astrocytes, but less efficiently from astrocytes-to-neurons. Interestingly, α-syn puncta are mainly found inside the lysosomal compartments of the recipient cells. However, differently from neurons, astrocytes are able to efficiently degrade fibrillar α-syn, suggesting an active role for these cells in clearing α-syn deposits. Astrocytes co-cultured with organotypic brain slices are able to take up α-syn fibrils from the slices. Altogether our data support a role for astrocytes in trapping and clearing α-syn pathological deposits in PD.

Keywords: α-synuclein, intercellular spreading, primary cultures, organotypic cultures, Parkinson's disease.

Introduction

The misfolding and aggregation of specific proteins is a common neuropathological hallmark of many neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's and prion diseases [39]. Parkinson´s disease (PD), a neurodegenerative disorder related to aging, is characterized by intracellular deposits of aggregated α-synuclein (α-syn) known as Lewy bodies and Lewy neurites [12]. In PD patients, at late stages of disease, deposits of α-syn aggregates are widely spread in the central nervous system (CNS) [11]. Several studies have shown that Lewy bodies may propagate within the brain [29, 32]. Evidences for the propagation of preformed α-syn aggregates through a prion-like process have been brought [1, 6, 42, 46]. The data obtained *in vitro* and *in vivo*, strongly suggest that cell-to-cell propagation of α-syn aggregates contribute to the spread of the pathology [16, 26].

Multiple lines of research are studying potential pathways for α-syn propagation not only from neuron-to-neuron but also between neurons and glial cells [3, 7, 19, 21, 31, 47, 50]. However the exact underlying route(s) allowing the physical movement of the protein aggregates from one cell to another as well as the cellular players involved in this process, are not yet fully understood. These issues are of paramount importance mainly from a therapeutic point of view, and need to be addressed [45].

In a previous study we reported that neurons efficiently internalise fluorescent α-syn fibrils, direct them to the lysosomal compartment and eventually transfer them to other neurons [1]. Furthermore, overexpressed α-syn is transferred from neuroblastoma SH-SY5Y cells to primary astrocytes *in vitro,* causing an inflammatory response in astrocytes [31]. However, the specific role of astrocytes in α-syn pathology is not clear and no study has quantitatively assessed yet the transfer from primary neurons to astrocytes and vice versa.

Astrocytes are the most abundant glial cell type in the CNS and are crucial for maintaining brain homeostasis and the correct functioning of neurons, oligodendrocytes and microglia [9, 33]. Astrocyte dysfunction alone can lead to neuronal degeneration and cell death [20, 34]. Under pathological conditions, astrocytes become reactive, upregulating pro-inflammatory genes, releasing cytokines and recruiting microglia among others, in order to promote neuronal survival [4, 57]. However, the release of inflammatory mediators by activated astrocytes could also induce pro-inflammatory activation in neighbouring neurons and other glial cells, contributing to progressive loss of neuronal function [15]. This suggests a dual role of astrocytes; in one hand perpetuating α-syn related-toxicity while in the other, providing neuroprotection against protein aggregates [15]. As the beneficial or detrimental role of astrocytes in PD is a current topic of controversy, here we aim to unravel the role of astrocytes in the intercellular spreading of fibrillar α-syn and to describe the intracellular fate of the assemblies in these cells.

To study neuron-astrocyte transfer of α-syn fibrils, we used *in vitro* (primary neuronal and astrocytic cultures) and *ex vivo* (organotypic hippocampal slices) models. Our data indicate an efficient transfer rate of α-syn from neurons-to-astrocytes and from astrocytes-to-astrocytes, whilst the efficiency of α-syn transfer from astrocytes-to-neurons was low. We show that fluorescent α-syn puncta are localized in the lysosomal compartment in astrocytes and neurons after internalization and also after transfer. In both cell types we detected a cleavage of the protein. However, while in neurons a progressive decrease of α-syn full-length protein over time was accompanied by a progressive increase of a cleavage product, astrocytes degraded quite efficiently both α-syn full-length and cleaved fragment.

In summary, the data presented here shed light on the role that astrocytes might have in the pathological mechanisms underlying synucleinopathies, highlighting their ability to take up and transfer α-syn fibrils between themselves, but less efficiently to neurons, and their ability to efficiently degrade α-syn aggregates.

Materials and Methods

Preparation of α-syn fibrils

For fibril formation, recombinant full-length, wild-type human α-syn, purified as described previously [24], was incubated in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM KCl) at 37 °C under continuous shaking in an Eppendorf Thermomixer set at 600 r.p.m. [10]. Fibrillar α-syn was centrifuged twice at 15,000 *g* for 10 min, resuspended twice in PBS and labeled with ATTO-550 (ATTO-Tec Gmbh) and Alexa Fluor 488 (Invitrogen, Carlsbad, CA) NHS fluorophore following the manufacturer's instructions using a protein:dye ratio of 1:2 as previously shown [10]. ATTO-550 or Alexa Fluor 488 labeled α-syn fibrils were fragmented for 15 min at 30 °C in 2 ml Eppendorf tubes in a VialTweeter powered by an ultrasonic processor UIS250v (250 watts, 24 kHz, Hielscher Ultrasonic, Teltow, Germany) set at 75% amplitude, 0.5 s pulses every 1 s. The nature of fibrillar α-syn forms before and after fragmentation was assessed using a JEOL 1400 transmission electron microscope following adsorption onto carbon-coated 200-mesh grids and negative staining with 1 % uranyl acetate.

Internalization assay of α-syn in neurons, astrocytes and organotypic hippocampal slices

Primary cultures of neurons and astrocytes and organotypic slices were prepared as described in the supplementary materials and methods (Online Resource 1). Cultured

neurons, astrocytes and organotypic slices were treated with 1µM of Alexa-488 or ATTO-550 fluorescent-tagged human recombinant α-syn fibrils. Depending on the experiment, we used either Alexa-488 or ATTO-550 labelled-fibrils, since they have the same properties and no differences were observed between them. Immediately before the internalization experiments, fibrils were diluted in the appropriate medium for each culture and sonicated for 5 min at 80 % amplitude with a pulse cycle of 5 s on and 2 s off in an ultrasonic water bath Vibra-Cell 75041 (Bioblocks Scientific). Internalization was evaluated after 16 h, or at different time points, after the cells were washed and culture medium was replaced. Then, primary and organotypic cultures were used for co-culture experiments, processed for immunofluorescence or western blot or the medium was collected for viability measurements.

Super-resolution structured illumination microscopy (SR-SIM)

Astrocytes were cultured on glass coverslips with a thickness of 1.5H (Marienfeld Superior). Immunofluorescence was performed as previously described for confocal microscopy using ProLong Gold as mounting media (ThermoFisher Scientific). SR-SIM was performed on a Zeiss LSM780 Elyra PS1 microscope (Carl Zeiss, Germany) using 63x/1.4 oil Plan Apo objective with a 1.518 refractive index oil (GE healthcare life science) and an EMCCD Andor Ixon 887 1K camera for the detection. 15 images per plane per channel (five phases, three angles) were acquired with a Z-distance of 91 nm to perform SR-SIM images. Acquisition parameters were identical for GFAP, α-syn fibrils and Lamp1 between control and ATTO-550 α-syn fibrils (1µM). SIM images were processed with ZEN software and then aligned with ZEN using 100 nm TetraSpeck microspheres (ThermoFisher Scientific) embedded in the same conditions as the sample. SIMcheck plugin in imageJ [8] was used to analyze the acquisition and the

processing in order to optimize between resolution/signal to noise ratio/artifacts. The images of SR-SIM are maximum intensity projections of 17 Z-stacks per condition. Brightness/Contrast was applied identically between conditions and thresholding was made on the mode value of the histogram of α-syn fibrils.

Co-culture systems of primary cells

Transfer experiments between donor astrocytes and acceptor astrocytes. In order to obtain two different cell populations, primary astrocytes were challenged overnight with 1 µM of ATTO-550 α-syn sonicated fibrils. The following morning, in order to label the acceptor population, naive astrocytes were incubated in suspension in a test-tube rotator (Labinco B.V.) for 30 min with CellTracker Green (CTG; ThermoFisher Scientific), 12.5 µM diluted in serum free medium, at 37 °C and with constant slow rotation. Then, the tube was centrifuged at 1000 r.p.m. for 10 min and the pellet washed 3 times with serum-free medium to completely remove the dye. Finally, the acceptor cells were resuspended, counted and mixed (ratio 1:1) in suspension with the donor cells, which were previously washed with PBS (6 times), trypsinized and counted. After 72 h in co-culture, the astrocytes were washed 3 times with PBS, fixed and immunostained. For quantification of α-syn transfer in this co-culture model, at least 100 cells were analysed in each independent experiment (n=3).

Transfer experiments between donor astrocytes and acceptor neurons. Primary donor astrocytes were challenged overnight with 1 µM of Alexa-488 α-syn sonicated fibrils. The following morning, donor astrocytes were washed 6 times with PBS, detached, counted, resuspended in complete neuronal medium and plated on top of one-week old naive neurons (acceptor population). For this set up, the ratio of donor astrocytes-acceptor neurons was maintained at 0.7:1. Cells were maintained under these conditions

for 72 h and then were washed (3 times with PBS), fixed, stained and analyzed as in previous experiments. For quantification of α-syn transfer in this co-culture model, at least 100 cells were analysed in each independent experiment (n=3).

Transfer experiments between donor neurons and acceptor astrocytes One-week-old donor neurons were treated with 1 μM of ATTO-550 α-syn sonicated fibrils for 16 h. The following morning, donor neurons were washed 3 times with PBS, and then, naive astrocytes (acceptor population) that were previously trypsinized, counted and resuspended in complete neuronal medium, were plated on top of the donor neurons at a ratio of 0.7:1. Cells were maintained under these conditions for 72 h and then were washed (3 times with PBS), fixed, stained and analyzed. For quantification of α-syn transfer in this co-culture model, at least 100 cells were analysed in each independent experiment (n=3). For western blot analysis of α-syn levels, protein extracts were obtained after 3 and 6 days in co-culture.

Co-culture of organotypic hippocampal slices and primary astrocytes

For these experiments hippocampal slices from WT mice and 10-20 div cortical astrocytes obtained from ROSA$^{mT/mG}$ mice, were used. Co-cultures were maintained for 3 or 6 days and then analyzed by immunofluorescence.

Transfer experiments between donor astrocytes and acceptor slices. ROSA$^{mT/mG}$ astrocytes growing in 24-well culture dishes were exposed or not to 1 μM of Alexa-488 α-syn sonicated fibrils. After 16 h, untreated and treated ROSA$^{mT/mG}$ astrocytes were rinsed with PBS, trypsinized and counted. Then, 7-10 μL of complete astrocyte medium containing 1000 ROSA$^{mT/mG}$ astrocytes were added on top of each slice.

Transfer experiments between donor slices and acceptor astrocytes. Organotypic hippocampal slices were exposed or not to α-syn fibrils. A 7 μL drop of complete

organotypic culture medium alone or containing 1 µM of Alexa-488 α-syn fibrils, were added on top of each slice. After 16 h of incubation, the slices were carefully rinsed with PBS (3 times). Then, untreated ROSA$^{mT/mG}$ astrocytes growing in 25 cm^2 flasks, were rinsed with PBS, detached, and counted. On top of each slice 1000 cells were seeded.

Immunocytochemistry and optical confocal microscopy of primary cells

In all conditions analyzed, immunostaining of neurons and astrocytes alone or in co-culture was performed following the same protocol. At the end of the experiments, the cells were rinsed with PBS, fixed with 4 % paraformaldehyde for 20 min and then permeabilized and blocked for 1 h in blocking solution (PBS with 2 % BSA and 0.01 % Saponin). Then, cells were incubated overnight at 4 °C with primary antibodies diluted in blocking solution. After rinsing with PBS, antibody binding was detected with the respective Alexa-conjugated secondary antibody for 1 h at room temperature (dilution 1:500), and the nuclei were counterstained with DAPI (1:2500; Sigma Aldrich). Finally, sections were mounted using aqua-poly/mount (Polysciences). The antibodies used were: mouse anti-MAP-2 (1:500; Merck Millipore), rabbit anti-GFAP (1:500; Dako), rat anti-Lamp1 (1:500; BD biosciences), mouse anti-α-synuclein, Syn-1 (1:500; BD Biosciences), and rabbit anti-α-synuclein (1: 150; ab138501 Abcam). After fixation and immunostaining, images were acquired with an inverted laser scanning confocal microscope LSM700 (Zeiss), with a 100x or 63x objective (zoom 0.5). Images were acquired using the Zen acquisition software (Zeiss) and further processed with ICY software (Quantitative Image Analysis Unit, Institut Pasteur. http://icy.bioimageanalysis.org/). In all experiments, we acquired Z-stacks covering the whole volume of cells, either when they were alone or in co-culture. In the transfer experiments, in order to quantify the percentage of donor and acceptor cells containing

α-syn puncta, the Z-stack was divided into the lower and upper part, segmenting only donor or acceptor cells, when possible, and then projecting the maximum intensity of those slices, using the ICY software. This was done in order to only have the whole donor or acceptor cell volume and to focus on what was inside the cells. Quantification of images was performed manually scrolling through the slices of the Z-stack to identify the puncta that were located inside the cell body based on nucleus identification and proximity. Overlapping cells were excluded from the analysis. Whereas all the images showed in the figures of neurons and astrocytes alone are projections of the entire Z-stack, the orthogonal views and images showed of co-culture experiments correspond to projections of selected slices of the Z-stack.

Immunofluorescence and confocal microscopy of organotypic cultures

Cultured slices were fixed with 4 % paraformaldehyde for 30 min and rinsed with PBS. Then, the slices were cryoprotected by immersion in 10 % sucrose solution for 1 h, and stored in 20 % sucrose at 4 °C until staining. Slices were permeabilized by quickly freeze-thawing (3 times), and subsequently incubated for 4 h with a blocking solution containing 20 % BSA, 5 % goat serum and 0.1 % triton X-100 (all from Sigma Aldrich) in PBS, to reduce unspecific labeling. After that, the slices were incubated during 24 h with primary antibodies, rinsed in PBS and incubated for 4 h with Alexa-conjugated secondary antibodies. Primary antibodies included mouse anti-α-synuclein (1:250; Syn-1 from BD biosciences) and rabbit anti-α-synuclein (1:250; C-20 from Santa Cruz biotechnology), mouse anti-β-III-tubulin (1:250; Sigma Aldrich), rabbit anti-GFAP (1:500; Dako) and mouse anti-GFAP (1:500; ThermoFisher Scientific), rabbit anti-Iba-1 (1:250; Wako), rat anti-Lamp1 (1:250; BD biosciences), and rabbit anti-RFP (1:250; Rockland). Antibodies were prepared in a solution of 5 % BSA, 1 % goat serum and 0.1

% triton X-100 in PBS. Incubations with primary and secondary antibodies were performed at room temperature. Then, the slices were rinsed with PBS and incubated with a DAPI solution for 5 min to visualize nuclei. Finally, sections were mounted using aqua-poly/mount. Confocal images were acquired with a confocal microscope LSM780 (Zeiss) using a C Apo 40X/1.2 W DIC III water objective. Imaging was performed with a diode 405 nm for DAPI, an argon laser line for Alexa Fluor 488, a DPSS561 nm laser for Alexa Fluor 546 and a HeNe 633 nm laser for Alexa Fluor 633. Images were acquired using the Zen acquisition software and further processed with ICY software.

Statistical analysis

Statistical analyses and graphs were performed using the GraphPad Prism version 6 software. All the results are expressed as the mean ± SD. For comparisons between two groups the Student's t-test was used. Unless stated in the figure's legend, for comparisons between more than two groups, one-way ANOVA with Tukey's *post hoc* analysis was employed. In all cases, statistical significance was attributed when $p \leq 0.05$.

Results

Exogenous α-syn fibrils are internalized by cortical astrocytes

In order to analyse the role of astrocytes in the spreading of α-syn fibrils we set up co-cultures between primary neurons and astrocytes. First we characterized α-syn fibrils internalization and subcellular localization in the two cell types separately. Primary cortical neurons were challenged with 1 μM of fluorescently labelled α-syn fibrils as shown before [1]. Internalization of α-syn fibrils was measured by western blots of neuron lysates after overnight incubation (Online Resource 2a). We also

assessed cell viability by measuring LDH release into the culture medium for up to 12 days, and found increasing levels of LDH release over time. However, we did not find significant differences between control neurons and neurons exposed to α-syn fibrils (Online Resource 2b). This suggested that α-syn fibrils are not toxic to neurons in culture within the timeframe of the experiment, in agreement with previous findings [1]. Moreover, we confirmed that the internalized α-syn co-localizes with the lysosomal marker Lamp1 (Online Resource 2c), differently from the endogenous soluble protein that is mainly excluded from lysosomes (Online Resource 2d).

Astrocytes have been shown to internalize α-syn aggregates [30]. Here we used 1 μM of ATTO-550 α-syn fibrils directly added to the medium of primary cortical astrocytes for 16 h. The fibrils were efficiently taken up by astrocytes, and fluorescent α-syn puncta were detected throughout the astrocytic cell body, however we observed a higher density of puncta in the perinuclear zone (Fig. 1a). Similar to what occurs in primary neurons, quantitative analysis revealed that 74.84 ± 1.3 % of α-syn puncta co-localize with Lamp1 after overnight exposure to fibrils (Fig. 1a). A more detailed analysis using super-resolution structured illumination microscopy (SR-SIM) revealed that α-syn puncta are located within the lysosomal vesicles (Fig. 1b). Similar to what occurs in neurons, we detected low levels of LDH release in cells exposed or not to fibrillar α-syn, indicating that α-syn fibrils are not affecting the viability of astrocytes within the experiment timeframe (Online Resource 3a). Several studies have reported that α-syn is not expressed (or is weakly expressed) in astrocytes [14, 30]. In agreement with those reports, in control astrocytes we did not detect the endogenous protein by western blot (Online Resource 3b) or immunofluorescence (Online Resource 3c). On the contrary, α-syn signal was revealed quite easily by western blot in astrocytes after exposure to human α-syn fibrils for 16 h (Online Resource 3b).

α-Syn fibrils transfer efficiently between primary astrocytes

In our previous study, we demonstrated that neuron-like CAD cells and primary cortical neurons propagate α-syn fibrils to other cells trough tunneling nanotubes (TNTs) [1, 2]. In order to assess the role of astrocytes in α-syn spreading, we first tested whether astrocytes were able to transfer α-syn fibrils between them. Thus, astrocytes were first exposed overnight to ATTO-550 labelled-α-syn fibrils. Then, after washing, the cells were detached and co-cultured with acceptor CellTracker Green (CTG)-labelled astrocytes for 72 h (Fig. 2a). Transfer of α-syn was measured by immunofluorescence and quantitative confocal microscopy. The entire cell population in the co-culture was labelled with the astrocytic marker GFAP, however because acceptor astrocytes were pre-labelled in green with CTG, this population could be easily distinguished from the donor astrocytes. After 72 h in co-culture, we observed that both donor and acceptor cells contained α-syn puncta (Fig. 2b). Z-stack imaging and orthogonal projection of these cells confirmed the presence of α-syn puncta within the acceptor cells (Fig. 2b). Under these experimental conditions, we calculated that 99.0 ± 0.7 % of donor cells and 75 ± 6.0 % of acceptor astrocytes contained α-syn puncta (Fig. 2c). This indicates that ATTO-550 α-syn fibrils could be efficiently transferred from donor to acceptor astrocytes in co-culture. In addition, and similar to what we found in neurons, we could show that the majority of the transferred α-syn puncta are within the lysosomal compartment of acceptor cells (Fig. 2d).

α-Syn fibrils transfer between primary cortical astrocytes and neurons

Astrocytes have a primordial role in various CNS processes and are fundamental for the correct functioning of neurons, oligodendrocytes and microglia [33]. Transfer of

α-syn has been already observed between SH-SY5Y cells and primary astrocytes *in vitro* [31]. This process, that may alter the homeostasis of the brain microenvironment, suggests that astrocytes might be perpetuating α-syn related-toxicity, implying that astrocytes play a role in non-cell autonomous spread of α-syn pathology. In order to shed light on the role of astrocytes in α-syn propagation, we performed a set of experiments to assess the transfer of fibrils between primary neurons and astrocytes and determine whether transfer is unidirectional or bidirectional. To this aim, we set up a co-culture model in which donor astrocytes, loaded with ATTO-550 α-syn fibrils for 16 h and then washed, were grown on top of naive cortical acceptor neurons (Fig. 3a). Transfer of α-syn puncta from donor astrocytes to acceptor neurons was evaluated after 72 h by immunofluorescence. Identification of donor astrocytes (GFAP positive) and acceptor neurons (MAP-2 positive) was made by acquiring Z-stacks covering the entire volume of both GFAP and MAP-2 positive cells, and segmenting the stacks into the lower and upper levels for analysis (Fig. 3b). With this method, we calculated that whilst 95.2 ± 3.7 % of the astrocytes still had α-syn puncta, 22.3 ± 7.3 % of acceptor neurons contained at least one α-syn puncta (Fig. 3c and 3d). Noteworthy, the number of puncta found per acceptor neuron was very low in comparison with the number detected in donor astrocytes. Thus, altogether these data indicate that transfer of α-syn from astrocytes to neurons can occur, but is very inefficient.

Next, we co-cultured naive acceptor astrocytes on top of donor neurons that were pre-exposed overnight to fluorescent α-syn fibrils, and thoroughly washed before receiving the acceptor cells (Fig. 4a). Donor and acceptor cells were identified by MAP-2 and GFAP staining of neurons and astrocytes, respectively. Analysis of transfer was made by confocal acquisition of Z-stacks covering the volume of donor neurons and acceptor astrocytes, followed by segmentation into donor and acceptor cells for analysis

(Fig. 4b and Online Resource 4). Quantitation of transfer showed that fibrillar α-syn puncta transferred from neurons to astrocytes (Fig. 4c), with 78.4 ± 19.4 % of acceptor astrocytes and 93.5 ± 0.6 % donor neurons containing α-syn puncta after 72 h co-culture (Fig. 4d) indicating that transfer from neurons to astrocytes was very efficient. Confocal analysis of acceptor astrocytes in co-culture for 72 h with donor neurons indicated that the transferred α-syn puncta were mainly localized in the lysosomes (Fig. 4e), similar to what we had previously shown in the case of interneuronal transfer [1]. From the data shown in Fig. 3 and Fig. 4 we conclude that fibrillar α-syn puncta transfer efficiently from neurons to astrocytes but not the other way round. This strongly suggests that transfer occurs preferentially in one direction.

α-Syn fibrils cleavage and degradation by neuron and astrocytes

The inefficient transfer of α-syn from astrocytes to neurons may suggest a role for astrocytes in α-syn trapping and/or degradation rather than in spreading. To study the fate of exogenously added α-syn fibrils in primary neurons and astrocytes, we performed time course experiments and analyzed protein levels and processing patterns by western blot. In pure primary cortical neurons, α-syn protein levels were measured for up to 9 days after overnight uptake (0 days) of ATTO-550 α-syn fibrils, followed by washes and addition of fresh medium to the cultures. In these conditions, we found a progressive decrease of the full-length protein (~16 kDa) in the neurons exposed to α-syn fibrils over time (Fig. 5a). Interestingly, along with the decrease in the full-length protein we consistently detected a cleavage product of α-syn (band ~14 kDa), which significantly increased over time and remained high in the treated cells until the end of the experiment (Fig 5a). When we calculated the ratio between the full-length and the lower molecular weight bands over time, we found that the ratio significantly

changed from 0.8 to 0.3 for the full-length band, and from 0.2 to 0.7 for the cleaved product, after 9 days (Fig 5b). We also measured α-syn protein levels in control and α-syn-loaded astrocytes after overnight (0), 6, 9 and 12 days. While we could detect α-syn cleavage in astrocytes exposed to α-syn fibrils, here there was no accumulation of the cleaved product over time (Fig. 5c). Furthermore, full-length α-syn was rapidly processed in astrocytes as compared to neurons. The amount of total α-syn (considering both the ~16 kDa and ~14 kDa bands) dropped to less than 10 % of the input in astrocytes after 6 days (Fig. 5d) as opposed to neurons at same time of culture (Fig. 5a). In order to test whether the distinct pattern in α-syn processing between neurons and astrocytes were due to differences in the rate of α-syn secretion, we analyzed by western blot the presence of α-syn in the culture medium of neurons and astrocytes exposed or not to α-syn fibrils up to 10 days. When compared to controls (0 days), we found a strong α-syn signal in the medium of both neurons and astrocytes after overnight exposure to fibrils. However at 3, 6 and 9 days post exposure, the release of α-syn in the medium from neurons was almost undetectable, while we detected low levels of α-syn in the medium from astrocytes (Online Resource 5a and 5b). These data suggest that degradation rather than a faster or massive release of α-syn by astrocytes is responsible for the differences in the protein levels observed between these cells.

Next, in order to confirm the source of the cleaved product observed in both neurons and astrocytes, we performed time course studies using an antibody (4B12) that specifically recognizes human α-syn in cells exposed or not to the human α-syn fibrils. In these conditions we observed the same pattern of processing in both cell types as detected using the Syn-1 antibody. We were also able to detect the α-syn cleaved product, confirming that the fragment originates from exogenous human fibrillar α-syn (Fig. 5e and 5f).

α-Syn fibrils degradation in neuron-astrocyte co-cultures

The data presented above indicate that human fibrillar α-syn resists degradation for several days within cortical neurons, whilst it is efficiently degraded in astrocytes. Therefore, we sought to determine what happens in co-culture conditions where both cell types are present. Indeed, given that α-syn fibrils transfer more efficiently from neurons to astrocytes than the reverse, we expected a reduction of the α-syn levels with time in a co-culture of α-syn-loaded neurons and naive astrocytes. This is exactly what we observed. Instead of detecting full-length and truncated α-syn as in the pure neuronal cultures (Fig. 5a), here a strong reduction of the full-length α-syn could be detected after 3 days of co-culture (Fig. 5g). Furthermore at day 6, the fraction of truncated α-syn was also substantially decreased (Fig. 5g). Moreover, we verified that both the full-length and cleaved fragment detected in the co-culture system correspond to human α-syn (Fig. 5h). These results strongly suggest that in the co-culture conditions, astrocytes rather than neurons are responsible for the reduction in the levels of exogenous α-syn. Finally, we corroborated by immunofluorescence using a human specific α-syn antibody that the transferred fluorescent puncta from neurons to astrocytes correspond to the human recombinant protein (Online Resource 5c). Altogether, these data suggest that neurons can transfer α-syn to astrocytes, where it is further degraded.

Internalization of exogenous α-syn fibrils by organotypic hippocampal slices

Next, we attempted to assess α-syn propagation in a more physiological context using an *ex vivo* system of organotypic hippocampal cultures. First we examined which cell types express endogenous α-syn by performing immunostaining of neurons (with

the axonal marker β-III-tubulin) and astrocytes (GFAP). As shown in the Online Resource 6a, α-syn is expressed by hippocampal neurons of the CA1 region. Importantly, we did not detect α-syn expression in the astrocyte population of the hippocampal slices (Online Resource 6b), which is in agreement with our *in vitro* results showing undetectable levels of endogenous α-syn in primary cortical astrocytes (Online Resource 3b and 3c) and with previous data [30]. In contrast to what was reported before for the endogenous distribution of α-syn in the adult mouse hippocampus [52], we also found α-syn diffuse expression in the cell body of a population of neurons located in the pyramidal layer of the CA1 region (Online Resource 6c). The same pattern of α-syn expression was detected by using two different antibodies against α-syn (Syn-1 and C-20) (Online Resource 6c).

In order to test the ability of different cell types in the hippocampal slices to take up exogenous α-syn fibrils, organotypic cultures were exposed for 16 h to 1 μM of ATTO-550 labeled α-syn fibrils. After rinsing the excess of α-syn an immunohistochemical analysis of the slices was performed to assess the cellular and subcellular localization of internalized α-syn. Interestingly, in these conditions we did not detect a significant internalization of labelled α-syn fibrils by neurons (Fig. 6a), while α-syn fibrils were efficiently taken up by astrocytes (Fig. 6b) and microglia (Online Resource 7a). Consistent with our data *in vitro*, the internalized α-syn puncta co-localize with Lamp1 in both astrocytes (Fig. 6b) and microglia (Online Resource 7b), as previously reported for overexpressed α-syn in transgenic mouse brains [36]. Co-localization analysis revealed that 40.5 ± 11.0 % of α-syn puncta co-localize with Lamp1 in loaded slices. A more detailed analysis revealed that α-syn puncta are located outside β-III-tubulin positive cells (Fig. 6a, right bottom panel) whilst they are found inside GFAP positive cells (Fig. 6b, right bottom panel).

Next, to assess the cytotoxic effects of α-syn internalization in the organotypic cultures, we quantified cell viability by measurement of LDH release in control and α-syn treated slices. After exposure to fibrillar α-syn, the slices were rinsed and further incubated up to 9 days, and the levels of LDH in the medium were measured at different time points. We found that exposure to α-syn fibrils does not affect cell viability at any of the time points assayed, since no significant differences were found between slices exposed or not to fibrillar α-syn (Online Resource 7c).

Transfer of α-syn fibrils between primary astrocytes and organotypic hippocampal slices in co-culture

To study the transfer of α-syn fibrils in organotypic cultures, we set up a co-culture system between organotypic hippocampal slices and primary astrocytes derived from $ROSA^{mT/mG}$ transgenic mice that express a cell membrane-localized red fluorescence in widespread cells/tissues [40]. Similar to previous reports showing that exogenous glial cells are able to integrate into the brain tissue [25, 27, 48], we found that after 3 days in co-culture, $ROSA^{mT/mG}$ astrocytes grow and extend processes inside the slices (Fig. 7).

To test whether astrocytes were capable of transferring α-syn fibrils to neurons or to other astrocytes in the organotypic slice, $ROSA^{mT/mG}$ astrocytes were pre-loaded with 1 μM Alexa-488 labelled α-syn fibrils for 16 h. Then, α-syn-containing astrocytes were washed and overlaid on top of naive organotypic hippocampal slices and co-cultured for 3 or 6 days (Fig. 7a). Although α-syn is degraded *in vitro* by astrocytes grown alone, a fraction of the cleaved form is still detectable after 6 days when astrocytes are co-cultured with neurons (Fig. 5g and 5h). Thus, we tested α-syn transfer from astrocytes to the slice for up to 6 days. As shown in Fig. 7b, transfer of α-syn fibrils

from the ROSA$^{mT/mG}$ astrocytes to the slice was detected as early as 3 days after co-culture onset. No α-syn signal was detected when the slices were co-cultured with control ROSA$^{mT/mG}$ astrocytes (Fig. 7b, top). Interestingly, transferred α-syn puncta were detected in GFAP positive cells (Fig. 7b, middle and bottom), while no α-syn puncta were detected in β-III-tubulin positive cells at any of the assayed time points (Fig. 7c, top and bottom). These findings indicate that transfer of α-syn fibrils principally occurs from astrocytes-to-astrocytes and not from astrocytes-to-neurons, which is consistent with what we found in our *in vitro* co-culture models (Fig. 2 and Fig. 3).

Moreover, we also observed transfer of α-syn fibrils from the slices to co-cultured astrocytes (Fig 8). In Fig. 8a, a schematic of the co-culture system is presented. Organotypic slices were loaded with 1 μM of α-syn fibrils labelled with Alexa-488, during 16 h. Then, the slices were rinsed to remove the excess of α-syn, and naive ROSA$^{mT/mG}$ astrocytes were laid on top. Co-cultures were maintained for 3 or 6 days. Transfer of α-syn from the hippocampal slices to naive astrocytes is shown in Fig. 8b.

Previously we found that exogenous α-syn fibrils co-localized with Lamp1 upon internalization by organotypic slices (Fig. 6). We investigated the subcellular localization of transferred α-syn in acceptor slices (Fig. 9a) or in acceptor astrocytes (Fig. 9b) using the two co-culture models described above. To determine the subcellular localization of α-syn puncta found either in the ROSA$^{mT/mG}$ donor astrocyte or in the acceptor slice, we analyzed i) the projection of 11 Z-stack images at the surface (P1) and at the center (P2) of the confocal Z-stack (Fig. 9a, left), ii) the 3D reconstruction of the entire Z-stack (Online Resource 8a) and, iii) the unstacked images of the Z-stack (Online Resource 8b). As shown in the right panels of Fig. 9a, both α-syn puncta present in α-syn-loaded ROSA$^{mT/mG}$ astrocytes (P1), and the puncta detected in the slice (P2), co-localize with Lamp1. Moreover, using a co-culture model where naive astrocytes were co-cultured

with α-syn-loaded slices, we found that α-syn puncta co-localize with Lamp1 in both the donor slice (S) and the acceptor ROSA$^{mT/mG}$ astrocytes (A) (Fig. 9b). Altogether, these data support the hypothesis that aggregated α-syn is normally directed to the lysosomal compartment in order to be degraded.

Discussion

The ability of misfolded α-syn to aggregate and spread throughout the brain has major implications for PD. However, there are still several unanswered questions regarding the cellular players involved in α-syn spreading and their role in disease progression. Because of the neurological nature of the disease, the propagation of α-syn has mainly been studied between neurons. In fact, several lines of evidence have already demonstrated neuron-to-neuron transfer of fibrillar α-syn [13, 23], yet not much is known about the contribution of astrocytes to this process [19, 21, 31, 50]. By setting different transfer assays *in vitro*, we have previously shown that α-syn fibrils transfer efficiently between neuron-like cells through TNTs [1]. Here we further implemented transfer assays both *in vitro* and *ex vivo*, to assess not the means of transfer (TNTs, secretion or other mechanisms [18]), but the cellular players involved in this transfer. We specifically studied the contribution of astrocytes to the intercellular spreading and degradation of α-syn. To our knowledge, this is the first time that α-syn transfer between primary cells and organotypic brain slices is evaluated. Recently it has been shown a bidirectional transfer of α-syn between neurons and astrocytes, however the efficiency of transfer was not quantified [17]. Our *in vitro* data demonstrate that cell-to-cell transfer of fibrillar α-syn occurs efficiently from neurons-to-astrocytes and between astrocytes, whilst is inefficient from astrocytes-to-neurons. These findings were validated *ex vivo,* in brain slices where transfer involved mainly astrocyte-to-astrocyte

interactions. In addition, we found that astrocytes are able to degrade α-syn fibrils more efficiently than neurons, where α-syn deposits remained for several days, thus suggesting that astrocytes could be limiting α-syn intercellular spreading to neurons. However, since a low rate of transfer from astrocytes to neurons was also observed, it can partially contribute to the propagation of fibrils. Moreover, it is possible that at longer terms the transferred α-syn fibrils (full-length and/or cleaved fragments) can seed the misfolded conformation of endogenous α-syn in the acceptor neurons, thus replicating the disease state. This possibility should be further evaluated in a specific study addressing seeding of transferred α-syn, which was not investigated here. Seeding of α-syn is a slow process that occurs inefficiently when the origin of the exogenous seeds is from a different species than the endogenous neuronal α-syn [35]. Therefore, to fully understand the possible pathological consequences of the transferred α-syn, seeding should be evaluated using cells and seeds of the same species.

α-Syn is a soluble protein of 140 amino acids, mainly localized in the presynaptic terminals and the nucleus of neurons [37]. In contrast to what has been reported, we found diffuse α-syn expression in the cell body of some pyramidal cells [52]. This discrepancy could be due either to culture conditions or to differences in the expression pattern of the protein as the neurons mature. Indeed, in primary neuronal cultures we observed an increase in the levels of endogenous α-syn over time. On the other hand, previous studies have reported that astrocytes, like other glial cells, do not express or express very low levels of endogenous α-syn [30, 47]. Our study confirmed that only neurons express α-syn to a detectable level both *in vitro* and *ex vivo*. This is relevant for the disease propagation, since it was shown that large amounts of endogenous α-syn constitutes a risk factor for intracellular aggregation [38, 51, 52, 54]. The fact that we detected abundant expression of α-syn in neurons compared to astrocytes, suggests that

neurons are more susceptible than astrocytes to form intracellular aggregates after exposure to exogenous α-syn fibrils. This also implies that the α-syn puncta found in astrocytes most probably come from another source. Indeed, *in vivo* data have demonstrated that induced formation of α-syn inclusions in glial cells occurred by transfer of α-syn from neurons [31].

It has been consistently reported that aggregated α-syn in neurons is directed to the lysosomal compartment for subsequent degradation [3, 5, 21, 22, 36, 49, 55, 56]. A fraction of α-syn assemblies appear to escape from the lysosomes [22], and seeding within the cytosol has been also reported [8]. Although α-syn fibrils were confirmed to be accumulating in the lysosomes of primary neurons [1], here we found a very inefficient degradation of α-syn fibrils by these cells. Instead, we observed a cleavage product of full-length α-syn that remained for over 9 days within the neurons. Evidence for such a degradation product has been brought previously [44] and it has been proposed that this α-syn cleaved band, is the result of proteolysis by kallikrein-6 serine protease [28, 41, 53]. Importantly, we previously identified a degradation product of exogenously applied fibrillar α-syn and showed it to be similar to processed α-syn within Lewy bodies [44]. Fibrillar α-syn processing occurred also in astrocytes. However, contrary to what happens in neurons, α-syn fibrils in astrocytes were almost completely degraded during the time course of the experiment. Importantly, when neurons pre-exposed to fibrils are co-cultured with astrocytes, we observed degradation of α-syn most likely by the acceptor astrocytes that received the fibrils. Taken together, all the aforementioned results support the hypothesis of a neuroprotective role of astrocytes against α-syn assemblies. In addition, they suggest that neurons generate an ~14 kDa fragment of α-syn that might play a role in the propagation of the pathology (either in the fibrillation process or in the spreading between neurons). However, more

studies are necessary to completely understand the mechanisms underlying this process.

Under a more physiological setting, using organotypic cultures we could not detect internalization of α-syn fibrils by neurons. However, we observed that α-syn fibrils were internalized and delivered to the lysosomal compartments of astrocytes and microglia, which is consistent with previous data showing that glial cells become reactive when an external stimulus is applied to the brain tissue [43]. Taken together, our data suggest that neuron-to-astrocyte propagation of α-syn fibrils is a normal process whereby astrocytes clear potentially toxic protein aggregates from neighbouring cells. In fact, we found that naive astrocytes are capable of taking up aggregated α-syn from the surrounding cells when they are plated on top of α-syn pre-loaded slices.

In organotypic cultures, when the slices were co-cultured with naive astrocytes we also found that α-syn is mainly transferred between astrocytes. Given that the transfer of α-syn fibrils preferably occurs from astrocyte-to-astrocyte and not from astrocyte-to-neuron, our results suggest cell type selectivity in the propagation of α-syn. Astrocytes might be exerting a neuroprotective role against intercellular transmission of α-syn aggregates. Because they do not express α-syn to a detectable level, amplification of the seeds within astrocytes is limited. Thus, astrocytes may act as traps in cell-to-cell spreading of the misfolded protein. However, it is also possible that increasing amounts of fibrillar α-syn deposits may ultimately have negative consequences for astrocytes and the surrounding cells. Indeed, a potent neuroinflammatory response of astrocytes has been described as a result of α-syn intercellular transfer from neuronal cells [31]. Also, a recent report showed that transfer of patient-derived α-syn aggregates from astrocytes to neurons leads to neuronal death [17].

In summary, the present study provides evidence of the involvement of astrocytes in the transfer and degradation of α-syn fibrils. By showing the existence of a cellular selectivity involved in the spreading of the aggregated α-syn, we have identified astrocytes as a key cellular player in this process. Therefore, we propose that astrocytes could be used as a target of novel therapeutic strategies for treating PD and other synucleinopathies.

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Figure legends

Fig. 1 Fluorescent α-syn fibrils are internalized by cortical astrocytes

a Representative Z-stack projections of confocal images showing primary cortical astrocytes in control conditions and after exposure to ATTO-550 α-syn fibrils for 16 h. Cells were labeled with anti-GFAP and anti-Lamp1 antibodies. The nuclei were counterstained with DAPI. The orthogonal cross-section views (x/y, x/z and y/z axes) correspond to one single slice of the Z-stack of the area marked with a yellow square in each condition. Yellow arrow shows co-localization between α-syn puncta and Lamp1. Scale bars represent 20 μm and 10 μm for the orthogonal view.

b Representative Z-stack projections of super-resolution structured illumination microscopy (SR-SIM) images showing primary cortical astrocytes in control conditions and after exposure to ATTO-550 α-syn fibrils for 16 h. Cells were labeled with anti-GFAP and anti-Lamp1 antibodies. The nuclei were counterstained with DAPI. The inset shows a magnified image of α-syn puncta and Lamp1 corresponding to the area marked with a yellow square in each condition. Scale bars represent 10 μm and 3 μm for the inset.

Fig. 2 α-Syn fibrils transfer efficiently between primary astrocytes

a Schematic of the experimental design of α-syn fibrils internalization and transfer between astrocytes. Donor astrocytes pre-exposed to ATTO-550 α-syn fibrils were thoroughly washed, detached and co-cultured with CellTracker Green (CTG)-labelled acceptor astrocytes (labelled in suspension) for 72 h. Then, the cells were fixed, stained and imaged.

b Representative confocal image showing a Z-stack projection of an expanded field of donor (**D**) and acceptor (**A**) astrocytes in co-culture after 72 h. Donor astrocytes were loaded with α-syn fibrils prior to co-culture with CTG-labelled acceptor astrocytes. The

cells were labelled with anti-GFAP and nuclei counterstained with DAPI. The smaller panels on the right show individual channel images of the same field for GFAP, α-syn fibrils and CTG positive cells. Intracellular localization of α-syn puncta in acceptor astrocytes (yellow arrow) was confirmed with the magnified orthogonal cross-section view (x/y, x/z and y/z axes) shown in the lower right corner panel that corresponds to a single slice of the Z-stack. Scale bars represent 50 μm and 10 μm for the orthogonal view.

c Bar graph showing the percentage of donor (light grey bar) and acceptor (dark grey bar) astrocytes containing α-syn puncta after 72 h in co-culture. Data show mean ± SD of 3 independent experiments.

d Representative confocal images of acceptor astrocytes after 72 h in co-culture. Donor astrocytes were loaded with α-syn fibrils prior to co-culture with CTG-labelled acceptor astrocytes. The cells were labelled with anti-Lamp1 and the nuclei were counterstained with DAPI. The orthogonal cross-section view (x/y, x/z and y/z axes) corresponds to one single slice of the Z-stack. Yellow arrow shows co-localization between α-syn puncta and Lamp1 in acceptor astrocytes. Scale bars represent 10 μm.

Fig. 3 α-Syn fibrils transfer from primary astrocytes to neurons

a Experimental set up used for evaluating astrocyte-to-neuron transfer of α-syn fibrils. Donor astrocytes pre-exposed to ATTO-550 α-syn fibrils for 16 h were thoroughly washed, detached and added on top of naive acceptor cortical neurons. After 72 h in co-culture the cells were fixed, stained and imaged.

b Representative confocal images showing projections of selected slices of the Z-stack covering the whole volume of donor astrocytes (upper panels) and acceptor neurons (bottom panels) after 72 h in co-culture. Donor astrocytes were exposed to ATTO-550 α-

syn fibrils before co-culturing them with naive neurons. The cells were labelled with anti-GFAP to identify astrocytes (white), anti-MAP-2 to identify neurons (green), DAPI to identify nuclei (blue), and the fibrils are red. Yellow arrows point to α-syn puncta localized within the soma of an acceptor neuron (MAP-2 positive). **D1**: donor astrocyte 1, **A1**: acceptor neuron 1, as indicated in Fig. 3c (left panel). Scale bars represent 10 μm.

c Representative confocal image showing the Z-stack projection of an expanded field showing donor astrocytes (**D**) and acceptor neurons (**A**) in co-culture after 72 h. The smaller panels on the right show individual channel magnified images of the same field for α-syn fibrils, GFAP, and MAP-2 of the area marked with a yellow square on the left panel. Yellow arrows point to α-syn puncta localized inside the soma of a second acceptor neuron (MAP-2 positive). D2: donor astrocyte 2, A2: acceptor neuron 2. Scale bars represent 50 μm and 10 μm for the inset.

d Bar graph showing the percentage of donor astrocytes (light grey bar) and acceptor neurons (dark grey bar) containing α-syn puncta after 72 h in co-culture. Data show mean ± SD of 3 independent experiments.

Fig. 4 α-Syn fibrils efficiently transfer from primary neurons to astrocytes

a Schematic of the experimental design of α-syn fibrils internalization in neurons and transfer to astrocytes. For these experiments, donor neurons were challenged with fluorescent α-syn fibrils (either Alexa-488 or ATTO-550) for 16 h and washed 3 times with PBS. Then, primary naive acceptor astrocytes maintained in parallel were detached, counted, plated on top of the donor neurons and kept in co-culture for 72 h.

b Representative confocal images showing projections of selected slices of the Z-stack showing donor neurons (upper panels) and acceptor astrocytes (bottom panels) after 72 h in co-culture. Donor neurons were loaded with Alexa-488 α-syn fibrils before co-

culturing them with naive astrocytes and, after being fixed, the cells were labelled with anti-GFAP, anti-MAP-2 and DAPI. Scale bars represent 10 μm.

c Representative image of the Z-stack projection corresponding to an expanded field showing donor neurons and acceptor astrocytes in co-culture after 72 h. The smaller panels on the right show magnified images of the area marked in yellow on the left panel, corresponding to individual channels of MAP-2, GFAP, and Alexa-488 fibrils. Intracellular localization of α-syn puncta in acceptor astrocytes (yellow arrow) was confirmed with the orthogonal cross-section view (x/y, x/z and y/z axes) presented in the lower right corner. Scale bars represent 10 μm.

d Bar graph showing the percentage of donor neurons (light grey bar) and acceptor astrocytes (dark grey bar) containing α-syn puncta after 72 h in co-culture. Data show mean ± SD of 3 independent experiments.

e Representative images of neurons and astrocytes in co-culture after 72 h. Donor neurons (unlabelled, indicated with orange arrows) were exposed to ATTO-550 α-syn fibrils prior to their co-culture with naive acceptor astrocytes. After being fixed, the cells were labelled with anti-Lamp1, anti-GFAP and DAPI. The orthogonal cross-section view (x/y, x/z and y/z axes) corresponds to one single image of the area marked with a yellow square, and shows co-localization between α-syn puncta and Lamp1 in acceptor astrocytes (yellow arrow). Scale bars represent 50 μm and 10 μm for the orthogonal view.

Fig. 5 α-syn fibrils processing and degradation

a Representative immunoblot of primary cortical neurons -/+ α-syn fibrils. Cell lysates from control and α-syn fibrils-loaded neurons were collected at different time points, and immunoblotted against α-syn (Syn-1) and α-tubulin (as a loading control).

b Bar graph showing the ratio between the full-length α-syn (light grey) and the α-syn breakdown product (dark grey), with respect to the total amount of protein for each time point. Data represent the mean ± SD of at least 3 independent experiments. A one-way ANOVA followed by Tukey's test was calculated for the full-length α-syn ratios ($F_{3,10}$= 26.07,p < .001), and for the cleaved fragment ratios ($F_{3,10}$= 26.07,p < .001) over time. ***p < 0.001 compared to overnight full-length, $^{###}p$ < 0.001 compared to overnight cleaved (0 days).

c Representative immunoblot of primary cortical astrocytes -/+ α-syn fibrils. Cell lysates from control and α-syn fibrils-loaded astrocytes were collected at different time points, and immunoblotted against α-syn (Syn-1) and GAPDH (as a loading control).

d Bar graph showing the quantification of the full-length α-syn (light grey) and the α-syn breakdown product (dark grey) as percentage of the control condition (overnight). Data represent the mean ± SD of 3 independent experiments. A one-way ANOVA followed by Tukey's test was calculated for full-length α-syn ($F_{3,8}$= 83.5,p < .001), and for the cleaved fragment ($F_{3,8}$= 17.89,p < .001) over time. ***p < 0.001 compared to overnight full-length, $^{###}p$ < 0.001, $^{##}p$ < 0.01 compared to overnight cleaved (0 days).

e Representative immunoblot of primary cortical neurons -/+ α-syn fibrils. Cell lysates from control and α-syn fibrils-loaded neurons were collected at different time points, and immunoblotted against α-syn (4B12; human specific) and α-tubulin (as a loading control).

f Representative immunoblot of primary cortical astrocytes -/+ α-syn fibrils. Cell lysates from control and α-syn fibrils-loaded astrocytes were collected at different time points, and immunoblotted against α-syn (4B12; human specific) and GAPDH (as a loading control).

g Representative immunoblot of cortical neurons -/+ α-syn fibrils co-cultured with naive astrocytes. Cell lysates from control and α-syn fibrils-loaded neurons were collected after overnight incubation, and after 3 and 6 days of being co-cultured with naive astrocytes. Immunoblots were performed against α-syn (Syn-1) and GAPDH (as a loading control).

h Representative immunoblot of cortical neurons -/+ α-syn fibrils co-cultured with naive astrocytes. Cell lysates from control and α-syn fibrils-loaded neurons were collected after overnight incubation, and after 3 and 6 days of being co-cultured with naive astrocytes. Immunoblots were performed against α-syn (4B12; human specific) and α-tubulin or GAPDH (as a loading control).

Fig. 6 Exogenous α-syn fibrils are efficiently internalized by astrocytes but not neurons in organotypic hippocampal slices

a Confocal images of double immunostaining for β-III-tubulin and Lamp1, in control (top panels) and ATTO-550 α-syn loaded hippocampal slices (bottom panels). The orthogonal cross-section views (x/y, x/z and y/z axes) correspond to one single slice of the Z-stack. The orthogonal views in the bottom panel shows that some of α-syn puncta (red) co-localize with Lamp1 (green) in α-syn loaded slices, but no α-syn puncta was detected in β-III-tubulin positive cells (crosshair). Yellow arrows point to α-syn puncta outside of a β-III-tubulin positive cell. The nuclei were counterstained with DAPI. Scale bars represent 20 μm.

b Confocal images of double immunostaining for GFAP and Lamp1, in hippocampal slices control (top panels) and ATTO-550 α-syn loaded hippocampal slices (bottom panels). The orthogonal cross-section views (x/y, x/z and y/z axes) correspond to one single slice of the Z-stack. Yellow arrows in the orthogonal views, point to α-syn puncta

(red) co-localizing with Lamp1 (green) in a GFAP positive cells. The nuclei were counterstained with DAPI. Scale bars represent 20 μm.

Fig. 7 Astrocyte-to-astrocyte transfer of α-syn fibrils in organotypic hippocampal slices

a Schematic of the co-culture system between donor ROSA$^{mT/mG}$ astrocytes and acceptor hippocampal slices. For these experiments, ROSA$^{mT/mG}$ astrocytes were challenged for 16 h with Alexa-488 α-syn fibrils. Then, the cells were detached, counted and plated on top of naive hippocampal slices for 3 or 6 days.

b Representative confocal images of control (upper panels), 3 days (middle panels) and 6 days (bottom panels) co-cultured slices, showing ROSA$^{mT/mG}$ donor astrocytes. Co-cultured slices were immunostained for RFP and GFAP. ROSA$^{mT/mG}$ astrocytes were labeled with an anti-RFP antibody to increase the specific red fluorescent signal. Also, GFAP staining show that ROSA$^{mT/mG}$ astrocytes are GFAP positive. No signal was detected in the green channel of control slices. The insets show a magnified image of the area marked in yellow in the respective merged panel. Yellow arrows point to α-syn puncta inside GFAP positive astrocytes of the slice. The nuclei were counterstained with DAPI. Scale bars represent 100 μm and 10 μm for the inset.

c Representative confocal images of 3 days (upper panels) and 6 days (bottom panels) co-cultured slices, showing ROSA$^{mT/mG}$ donor astrocytes. Co-cultured slices were immunostained for RFP and β-III-tubulin. The insets show a magnified image of the area marked in yellow in the respective merged panel. Yellow arrows point to α-syn puncta outside ROSA$^{mT/mG}$ donor astrocytes. No α-syn puncta was detected in the cell body of β-III-tubulin positive cells. The nuclei were counterstained with DAPI. Scale bars represent 100 μm and 10 μm for the inset.

Fig. 8 Astrocytes internalize α-syn fibrils from organotypic hippocampal slices

a Schematic of the experimental design. For these experiments, donor slices were challenged for 16 h with Alexa-488 α-syn fibrils and washed 3 times with PBS. Then, primary naive acceptor ROSA$^{mT/mG}$ astrocytes maintained in parallel were detached, counted and plated on top of the donor slices for 3 or 6 days.

b Representative confocal images of control (upper panels), 3 days (middle panels) and 6 days (bottom panels) co-cultured slices, showing ROSA$^{mT/mG}$ acceptor astrocytes. Co-cultured slices were immunostained for RFP and GFAP. The insets show a magnified image of the area marked in yellow in the respective merged panel, without showing GFAP signal. Yellow arrows point to α-syn puncta inside ROSA$^{mT/mG}$ acceptor astrocytes. The nuclei were counterstained with DAPI. Scale bars represent 20 μm and 10 μm for the inset.

Fig. 9 α-Syn is localized in lysosomes of donor and acceptor cells in *ex vivo* co-culture models

a Confocal Z-stack images of a hippocampal slice co-cultured during 3 days with α-syn-loaded ROSA$^{mT/mG}$ astrocytes. Co-cultured slices were immunostained for RFP (white) and Lamp1. 8 stacked images out of a 42-image series starting on 0.8 μm from the surface to 17.2 μm depth in the slice with 0.4 μm plane thickness are shown (left). The panels on the right show the Z-stack projections of the images comprised from 0.8 μm to 4.8 μm (indicated as P1) and from 8.4 μm to 12.4 μm (indicated as P2). The last panels display an orthogonal view of the area marked in yellow in the merged panel, showing only red and green channels. The orthogonal cross-section views (x/y, x/z and y/z axes) correspond to one single slice of the Z-stack. Yellow arrows point to α-syn puncta that

co-localize with Lamp-1. The nuclei were counterstained with DAPI. Scale bars represent 20 μm and 10 μm for the orthogonal views.

b Representative Z-stack projection of an expanded field showing donor slices (S) and acceptor astrocytes (A) co-cultured for 3 days (left). Co-cultured slices were immunostained for RFP (white) and Lamp1. The smaller panels on the right show a magnification of the areas indicated in the left image. The last panels display an orthogonal view of the area marked in yellow in the merged panel, showing only red and green channels. The orthogonal cross-section views (x/y, x/z and y/z axes) correspond to one single slice of the Z-stack. Yellow arrows point to α-syn puncta that co-localize with Lamp-1. The nuclei were counterstained with DAPI. Scale bars represent 100 μm (left image), 20 μm (right panels), and 10 μm for the orthogonal views.