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α-Synuclein transfer between astrocytes and neurons indicates that astrocytes play a role in degradation rather than in spreading

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Supplementary Materials and Methods Animals

C57BL/6 wild type (WT) and transgenic Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo} (hereafter named as ROSA^{mT/mG}) mice from our in-house colony (Institut Pasteur, Paris, France) were used. Animals were housed in cages with filter tops in a ventilated rack and maintained on food and water *ad libitum*. Handling of animals was performed in compliance with the guidelines of animal care set by the European Union and the French regulations on the use and care of laboratory animals, and approved by the local Pasteur Ethics Committee (CETEA; Comités d'étique en experimentation animale).

Primary astrocyte cultures

Astrocyte cultures were prepared from postnatal WT or transgenic ROSA^{mT/mG} mice (1-3 days after birth), as previously described [2]. Dissociated cortical cells (neocortex) were grown in 25 cm² flasks coated with 5 μ g poly-D-lysine (PDL) in complete astrocyte medium composed of DMEM-Glutamax[®] (Gibco) supplemented with 10% horse serum (Sigma Aldrich), 10% foetal bovine serum (Eurobio), and penicillin/streptomycin (Gibco). Cultures were kept at 37 °C in a humidified 5% CO₂-containing atmosphere until they reached confluency (7–10 days in vitro; div). Culture medium was refreshed every three days. To reduce proliferation of other glial cells a mixture of 5-Fluoro-2'-deoxyuridine / Uridine (10 μ g/ml; both from Sigma-Aldrich) was added to the culture for 24 h. To perform experiments, confluent cultures were detached with trypsin (Gibco) and passed onto multi-well plates with or without PDL-coated coverslips.

Primary neuronal cultures

Primary cortical neurons were prepared from WT mice at embryonic day 17. Briefly, after removing and decapitating the foetuses, the meninges were stripped off the brain. The cortices were removed and digested in a mixture of trypsin (0.25 %; Gibco) and DNaseI (1 mg/mL; Roche Diagnostics) for 15 minutes at 37 °C, and then dissociated by trituration with two fire-polished Pasteur pipettes of different diameter. The dissociated cells where then plated (45.000/cm²) in 4-well dishes with or without PLL-coated (1 mg/mL; Sigma Aldrich) coverslips in Neurobasal medium (Gibco) supplemented with 10

% horse serum. After 3 h the medium was changed to complete neuronal medium (Neurobasal medium supplemented with B-27, Glutamax[®] and penicillin/streptomycin, all from Gibco). Non-neuronal cell division was halted by exposure to 2 μ M cytosine-D-arabinofuranoside (Ara C; Sigma Aldrich) for 24 h. The cells were then maintained at 37 °C in a humidified atmosphere containing 5 % CO₂.

Preparation of organotypic hippocampal slice cultures

Slices preparation and culture were performed following a protocol based on the interface culture method [1, 3]. Briefly, hippocampal slices were prepared from WT mice (6-9 days after birth). Animals were decapitated and the brain was removed and immediately submerged in cold artificial cerebrospinal fluid (ACSF: 87 mM NaCl, 26 mM NaHCO₃, 50 mM sucrose, 10 mM glucose, 2.5 mM KCl, 1.25 mM NaH₂PO₄·H2O, 0.5 mM CaCl₂, 3 mM MgCl₂·6H₂O), bubbled with carbogen (95 % O₂, 5 % CO₂). Coronal slices from the hippocampus (350 μ m) were cut using a vibrating-blade microtome (Leica). Slices were transferred into cell culture inserts of 0.4 μ m pore size (Merk Millipore), and placed in 6-well culture dishes containing 1.2 mL of culture medium. Organotypic culture medium consisted of 50 % minimum essential medium (Gibco), 25 % Hanks balanced salt solution (Gibco), 24 % horse serum, 1 % penicillin-streptomycin, and supplemented with 36 mM D-glucose (Sigma Aldrich) and 25 mM Hepes (Gibco). Culture slices were maintained in a cell culture incubator at 37 °C at 5 % CO₂. The culture medium was replaced with fresh complete organotypic medium every two days. All the experiments performed with organotypic slices, started after 10 div.

Co-localization analysis in primary and organotypic cultures

The quantification of co-localization was made by using the Colocalizer V1 script developed for ICY software (http://icy.bioimageanalysis.org/protocol/Colocalizer). This protocol relies on object-based methodology and gives the percentage of co-localization based on the minimal distance between two objects. Briefly, in order to get the co-localization rate of the fibrils against Lamp1, the distance of the center of mass of each α -syn puncta against each Lamp1 spot was computed. They were considered co-localized if the distance was lower than 4 pixels. In addition, co-localization was confirmed with orthogonal cross-section views of selected single slices of the Z-stack showing x/y, x/z and y/z axes using also the ICY software.

Chloroform/methanol precipitation of culture media

Experiments were performed at 4 °C. Culture medium samples were collected and centrifuged at room temperature for 4 min at 1200 r.p.m. to eliminate cell debris. Then, supernatant was collected in a new tube and three volumes of methanol were added to one volume of the protein sample, and the mixture was vortexed. One volume of chloroform was then added, and the mixture was vortexed. After adding three volumes of ultra-pure H₂O and mixed by pulse-vortexing, the sample was centrifuged at 9 500 r.p.m. for 5 min and the aqueous methanol layer was removed from the top of the sample. The proteins remained at the phase boundary between the aqueous methanol layer and the chloroform layer. Three volumes of methanol were added, and the mixture was vortexed. The sample was centrifuged at 9 500 r.p.m. for 10 min. The supernatant was removed without disturbing the pellet, the pellet was air dried and then suspended in RIPA buffer (50 mM Tris-HCL pH 7.2, 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % NP40, 1 % sodium dodecyl sulfate (SDS) and a cocktail of protease inhibitors).

Western immunoblot in primary cells

For the time course experiments in primary neurons and astrocytes, as well as the neuron-to-astrocytes co-culture experiments, at the end of the experiment the cells were rinsed with PBS and then scraped in RIPA buffer. The resulting soluble fractions were centrifuged at 14000 r.p.m. for 5 min at 4 °C, and then the supernatants were taken as the whole cell lysate, mixed with Laemmli sample buffer (1 M Tris-HCl, pH 6.8, 20 % glycerol, 4 % SDS, 5 % β-mercaptoethanol and 0.02 % bromophenol blue) and boiled for 5 min. For western blots, equal amounts of proteins (5-10 μ g/lane) were separated on 14 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare Life sciences), following standard procedures. Membranes were then blocked in TBS-T; Tris-buffered saline containing 0.1 % Tween-20 and 5 % non-fat dried milk for 1 h, and probed overnight at 4 °C with primary antibodies. The membranes were then washed and exposed for 1 h at room temperature to the appropriate secondary peroxidase-conjugated antibody (1:5000). The specific protein bands were visualized using the ECL-immunoblotting chemiluminescence system (GE Healthcare Life sciences) and the ImageQuant LAS 500TM camera (GE Healthcare Life sciences). Primary antibodies used in this study were: mouse anti- α -synuclein (1:500; Syn-1 BD Biosciences), mouse anti- α -synuclein (1: 5000; 4B12 ThermoFischer Scientific) mouse anti- α -tubulin (1:5000; Sigma Aldrich), and rabbit anti-GAPDH (1:500; Boster). To determine the apparent molecular weights of the protein bands a PageRuler plus prestained protein ladder (ThermoFisher Scientific) was used.

Toxicity measurements

Cell death in neurons, astrocytes and organotypic brain slices was quantified by measuring the lactate dehydrogenase (LDH plus kit, Roche Diagnostics) release into the culture medium following the manufacturer's instructions. Briefly, the cells and slices were exposed to 1 μ M of α -syn fibrils, as described before. After washing and adding new medium, LDH release was measured in the cultured media at different time points. 100 μ l medium was incubated (in triplicates) with 100 μ l of the reconstituted substrate for up to 20 min at room temperature in darkness. Reaction was stopped by adding 50 μ l of stop solution, and absorbance was read at 490 nm using a Tecan microplate reader. Background absorbance due to culture medium alone was subtracted from all readings. Data are presented as optical density in arbitrary units (a.u.).

References

- Gogolla N, Galimberti I, DePaola V, Caroni P (2006) Preparation of organotypic hippocampal slice cultures for long-term live imaging. Nature protocols 1: 1165-1171 Doi 10.1038/nprot.2006.168
- 2 Loria F, Petrosino S, Hernangomez M, Mestre L, Spagnolo A, Correa F, Di Marzo V, Docagne F, Guaza C (2010) An endocannabinoid tone limits excitotoxicity in vitro and in a model of multiple sclerosis. Neurobiology of disease 37: 166-176 Doi 10.1016/j.nbd.2009.09.020
- 3 Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. Journal of neuroscience methods 37: 173-182

Online Resource 2



Exogenous α -syn fibrils are internalized and delivered to lysosomes of primary **neurons.** a Representative immunoblot of neurons $-/+ \alpha$ -syn fibrils. Cell lysates from neurons exposed overnight or not to fibrillar α -syn were collected and immunoblotted against α -syn and α -tubulin (as a loading control). The separated lane on the left shows the migration pattern of exogenous fibrils loaded without cells. The bar graph shows the quantification in control (light grey) and neurons exposed to α -syn fibrils (dark grey). Data show mean \pm SD of 3 independent experiments. ***p < 0.001 compared to CTL, Student's t-test. **b** Cell toxicity was measured by LDH release in neurons after 16 h (0 d), 3, 6, 9, and 12 days after exposure to α -syn fibrils. The graph represents the optical density (arbitrary units). There were no significant differences between neurons exposed (black circles) or not (black triangles) to fibrillar α -syn at any of the time points evaluated. Data show mean ± SD of 3 independent experiments. A two-way ANOVA was calculated ($F_{1,26}$ = 4.182,p = .051), ns= not significant. **c** Representative Z-stack projections of confocal photomicrographs showing primary neurons in control conditions and after exposure to ATTO-550 α -syn fibrils for 16 h. Cells were labeled with anti-MAP-2 and anti-Lamp1 antibodies. 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. The yellow arrow shows co-localization between α -syn puncta and Lamp1. Scale bars represent 10 μ m. **d** Representative Z-stack projection of confocal images showing primary neurons after exposure to ATTO-550 α -syn fibrils for 16 h. Cells were labeled with anti- α -syn (Syn-1) and anti-Lamp1 antibodies, 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. The yellow arrow shows the lack of co-localization between endogenous α -syn and Lamp1. Scale bars represent 10 μ m.



Detection of endogenous and exogenous α -syn in astrocytes. a Cell toxicity was measured by LDH release in astrocytes 16 h (0 d), 6, 9 and 12 days after exposure to α syn fibrils. The graph represents the optical density (arbitrary units). There were not significant differences between astrocytes exposed (black circles) or not (black triangles) to fibrillar α -syn at any of the time points evaluated. Data show mean ± SD of 3 independent experiments. A two-way ANOVA was calculated ($F_{1,50}$ = 1.276,p = .264), ns= not significant. **b** Cell lysates from astrocytes exposed overnight or not to ATTO-550 α syn fibrils (n=2) were collected and immunoblotted against α -syn (Syn-1) and α -tubulin (as a loading control). **c** Representative confocal images of the intracellular localization of endogenous α -syn in primary astrocytes stained with anti-GFAP and anti- α -syn (Syn-1). The nuclei were counterstained with DAPI. Scale bar represents 20 µm.



GFAP / MAP-2 / α-syn fibrils / DAPI

 α -Syn fibrils efficiently transfer from primary neurons to astrocytes. Unstacked Zstack images of neurons and astrocytes after 72 h in co-culture allows appreciating that neurons (MAP-2) and astrocytes (GFAP) are on different planes. Note that both donor neurons and acceptor astrocytes contain α -syn puncta. Scale bars represent 20 μ m.



Limited α -Syn release into the culture media of neurons and astrocytes. Detection of human α -syn fibrils in the culture medium of **a**, primary cortical neurons, and **b**, primary cortical astrocytes. Cells were exposed or not to ATTO-550 α -syn fibrils for 16 h, then thoroughly rinsed with PBS and the culture medium was replaced with a fresh one and cells were further incubated for 3, 6 and 10 days. Culture media were subsequently collected at different time points, the proteins were precipitated and equal volumes were loaded and resolved by SDS-PAGE (14 %), immunoblotted and probed with an antibody against α -syn (4B12: human specific). On the right panel, the Ponceau S stained blot is shown as loading control. **c** Representative confocal images showing acceptor astrocytes after being co-cultured with donor neurons (-/+ ATTO-550 α -syn fibrils) for 72 h. Cells were washed, fixed and labeled with anti-GFAP and anti- α -syn (ab138501, human specific). Nuclei were counterstained with DAPI. Scale bars represent 10 µm.



Endogenous expression pattern of α -syn in organotypic hippocampal slices. a Representative Z-stack projection of an expanded field of the CA1 hippocampal area. Slices were triple immunostained for β -III-tubulin, GFAP, and α -syn. Insets show a magnified image of the area marked in yellow in the merged panel. The yellow arrow points to α -syn in β -III-tubulin positive cells. The nuclei were counterstained with DAPI. Scale bars represent 100 μ m and 10 μ m for the inset. **b** Orthogonal views of the area marked in yellow in the merged panel of **a**, showing either red and green channels (left panel) or red and blue channels (right panel). The orthogonal cross-section views (x/y, x)x/z and y/z axes) correspond to one single slice of the Z-stack. Endogenous expression of α -syn in the cell body of a population of neurons in the pyramidal layer was recognized by using C-20 antibody while no signal of α -syn was observed inside of a GFAP positive cell (vellow arrows). Scale bars represent 10 µm. **c** Representative Z-stack projections of the CA1 hippocampal area. Intense immunoreactivity of α -syn (red) was detected as puncta and as a diffuse signal in the cell body of a population of neurons in the pyramidal layer, by using both Syn-1 (left panel) and C-20 (right panel) α -syn antibodies. The nuclei were counterstained with DAPI. Scale bars represent 100 µm.



 α -Syn fibrils are internalized by microglia in organotypic hippocampal slices. a Images of a hippocampal slice incubated with ATTO-550 labeled α -syn fibrils (1 μ M) immunostained for the microglial marker, Iba-1. The insets show a magnification of the area marked in yellow in the merged panel. The right panel shows an orthogonal view of the image depicted in the inset panel presenting only the 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 (red) inside of an Iba-1 positive cell (green). The nuclei were counterstained with DAPI. Scale bar represents 20 µm and 10 μ m for the inset. **b** In the slice exposed to fibrillar α -syn, most of the detected α -syn puncta (red) is associated with Lamp1 (green). Yellow arrow in the orthogonal view points to α -syn puncta co-localizing with Lamp1 in an Iba-1 positive cell (white). The nuclei were counterstained with DAPI. Scale bar represents 20 µm. c Cell death in organotypic hippocampal slices was assessed by measuring LDH release into the medium of control and α -syn-loaded slices. LDH release was measured at 0, 3, 6 and 9 days after exposure to α -syn fibrils. The graph shows the optical density variation (arbitrary units) with time. There were not significant differences between control (black triangles) and slices exposed to α -syn fibrils (black circles) at any of the time points evaluated. Data show mean \pm SD of 5 independent experiments. A two-way ANOVA was calculated ($F_{1,32}$ = 0.317,p = .577), ns= not significant.



b

RFP / α-syn fibrils / Lamp1 / DAPI

0.8 µm	1.2 µm	1.6 µm	2.0 µm	2.4 µm	2.8 µm	3.2 µm
3.6 µm	4.0 µm	4.4.µm	4.8 µm	5.2 µm	5.6° µm	6.0 µm
6.4 µm	6.8 µm	7.2 µm =	7.6 µm	а 8.0 µm	а 8.4 µm —	8.8 µm
9.2;µm* (-	9.6;µm*	10.0 ⁻ µm —	10.4 µm –	10.8 µm —	11.2 µm	11.6 µ́m —
12.0 µm. —	12.4 ⁻ µm —	12.8 µm –	13.2 μm –	13.6 µm	14.0 µm -	14.4 µm
14 Rum	15 2 um -	15.6 um -	16 0 um -	16.4 um	16 8 um	17 2 um

α-Syn fibrils co-localize with Lamp1 in donor astrocytes and acceptor slices. a Reconstructed 3D projection of the Z-stack images presented in Fig. 9a, showing ROSA^{mT/mG} donor astrocytes (white) co-cultured during 3 days with an acceptor slice. Yellow arrows show α-syn puncta (green) outside of the ROSA^{mT/mG} astrocytes. Scale bar represents 20 µm. **b** Unstacked Z-stack images of the series showed in Fig. 9a starting on 0.8 µm from the surface to 17.2 µm depth in the slice with 0.4 µm plane thickness. Yellow arrows point to α-syn puncta outside the ROSA^{mT/mG} astrocytes that are associated with Lamp1. Scale bar represents 20 µm.