

Unraveling the β -amyloid clearance by astrocytes: Involvement of metabotropic glutamate receptor 3, sAPP α , and class-A scavenger receptor

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ABSTRACT

The mechanics of β -amyloid (A β) clearance by astrocytes has not been univocally described, with different mediators appearing to contribute to this process under different conditions. Our laboratory has demonstrated neuroprotective effects of astroglial subtype 3 metabotropic glutamate receptor (mGlu3R), which are dependent on the secreted form of the amyloid precursor protein (sAPP α) as well as on A β clearance; however, the mechanism underlying mGlu3R-induced A β uptake by astrocytes remains unclear. The present study shows that conditioned medium from mGlu3R-stimulated astrocytes increased A β uptake by naïve astrocytes through a mechanism dependent on sAPP α , since sAPP α depletion from conditioned medium inhibited A β phagocytosis. Concordantly, recombinant sAPP α also increased A β uptake. Since we show that both sAPP α and the mGlu3R agonist LY379268 increased expression of class-A scavenger receptor (SR-A) in astrocytes, we next determined whether SR-A mediates mGlu3R- or sAPP α -induced A β uptake by using astrocyte cultures derived from SR-A knockout mice. We found that the effects of LY379268 as well as sAPP α on A β uptake were abolished in SR-A deficient astrocytes, indicating a major role for this scavenger receptor in LY379268- and sAPP α -stimulated A β clearance by astrocytes. We also show results of coimmunoprecipitation and functional assays offering evidence of possible heterotrimerization of sAPP α with A β and SR-A which could allow A β to enter the astrocyte. In conclusion the present paper describes a novel pathway for A β clearance by astrocytes involving sAPP α as an enhancer of SR-A-dependent A β phagocytosis.

1. Introduction

Metabotropic glutamate receptors (mGluR) belong to the superfamily of class III G-protein coupled receptors. Eight subtypes of mGluR have been described, classified into 3 groups. In Group II mGluR, the mGlu2R subtype is mainly presynaptic whereas the mGlu3R subtype is found preferentially in postsynaptic membranes and glial cells (Riedel et al., 2003; Schoepp, 2001). Neuron-glia interaction has been widely shown to promote neuroprotection, in part through mechanisms involving group II mGluR activation (Bruno et al., 2017).

Alzheimer's disease (AD) is the major cause of dementia for older

people. Whereas the early-onset, hereditary form of AD is considered to derive from increased production of β -amyloid (A β) peptide from amyloid precursor protein (APP), the late-onset AD is thought to arise after failures in A β clearance mechanisms (Mawuenyega et al., 2010; Gallina et al., 2015; McIntee et al., 2016).

At the cell surface, APP undergoes proteolysis by α -secretases that cleave it between Lys₆₈₇ and Leu₆₈₈ thereby releasing a large, soluble ectodomain: sAPP α . Alternatively, APP can be cleaved by β -secretase to generate a fragment which -after further cleavage by γ -secretase- yields the A β peptide. We recently showed that mGlu3R activation by the agonist LY379268 promotes the non-amyloidogenic (or 'alpha')

Abbreviations: mGlu3R, subtype 3 metabotropic glutamate receptor; APP, amyloid derived protein; sAPP α , secreted form of α -secretase-cleaved amyloid precursor protein; A β , β -amyloid; SR, scavenger receptors; SR-A, class-A scavenger receptor; AD, Alzheimer's disease; GCM, glial conditioned medium; IP, immunoprecipitation; IB, immunoblot

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cleavage of APP in cultured astrocytes, leading to increased release of sAPP α (Durand et al., 2014), which has been demonstrated to exert neuroprotective action in several injury models and to improve cognition in AD and aging (Habib et al., 2017). Furthermore, mGlu3R expression is diminished in hippocampal astrocytes from PDAPP-J20 mice (Durand et al., 2014), suggesting a role for this receptor in AD. In addition, our most recent results show that astrocyte-derived sAPP α protects hippocampal neurons from A β neurotoxicity (Durand et al., 2017). Moreover, LY379268 is also able to increase A β uptake in both astrocytes and microglia, an effect that impacts positively on the survival of co-cultured neurons (Durand et al., 2017). Nevertheless, how mGlu3R triggers A β engulfment by astrocytes is an unexplored research topic.

Glial cells are the main scavengers of the brain, and A β is primarily internalized through receptor-mediated endocytosis (Ries and Sastre, 2016). Scavenger receptors (SR), Toll-like receptors, receptors for advanced glycation end products (RAGE), Fc receptors, and lipoprotein receptor-related proteins (LRP), among others, may be involved in A β uptake (Ries and Sastre, 2016). In microglia, we showed involvement of CD11b in the recognition/endocytosis of A β induced by Group II mGluR activation (Durand et al., 2017), whereas in astrocytes the underlying mechanism remains to be studied. Although we previously showed that LY379268 upregulates gene expression of SR CD36 in astrocytes (Durand et al., 2017), the literature points to class A SR (SR-A) as the central A β -interacting receptor in AD models. SR-A expression is reduced in the hippocampus of aged and APP/PS1 animals (Cornejo et al., 2018), and a triple transgenic APP/PS1/SR-A^{-/-} mouse displays a reduced lifespan, reduced cognitive performance, and increased pro-inflammatory cytokine levels, as well as reduced microglial phagocytic capacity (Cornejo et al., 2018). Consequently, these triple transgenic mice show accelerated A β accumulation, and pharmacological upregulation of SR-A expression on mononuclear phagocytes increases A β clearance (Frenkel et al., 2013). Interestingly, an association between sAPP α and A β clearance has also been demonstrated: sAPP α can interact with several partners participating in the A β clearance mechanism, such as SR-A and LRP1 in microglia, neurons, peripheral monocytes, and endothelial cells from the blood-brain barrier (Habib et al., 2017). sAPP α enhances SR-A-mediated phagocytosis of A β by microglia and monocytes (Habib et al., 2017), and upregulates A β -degrading enzymes in microglia from APP/PS1 Δ E9 mice, thereby reducing A β plaques (Fol et al., 2016). However, involvement of sAPP α in astroglial A β clearance was not yet revealed. Our present work shows molecular mechanisms involved in enhanced A β removal by the mGlu3R/sAPP α system in astrocytes.

2. Methods

2.1. Animals

Wistar rat pups were obtained from the animal facility of INBIOMED Argentina. SR-A knockout and wild-type mice (129/ICR background) were kindly facilitated by their developer, Dr. Tatsuhiko Kodama (Research Center for Advanced Science and Technology, University of Tokyo, Japan) (Suzuki et al., 1997) and kept in the animal facility of the Neuroscience Department at School of Medicine, Pontificia Universidad Católica de Chile. Animals were housed in a temperature-regulated room with a 12 h light/dark cycle, with free access to food and water. Mice were genotyped after weaning.

Experimental procedures were carried out in accordance with the National Institute of Health guidelines for the care and use of laboratory animals, with approval by the Committee on Ethics of the University of Buenos Aires Medical School (Res. 2068/2015) and the Pontificia Universidad Católica de Chile School of Medicine. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Astrocyte primary cultures

Astrocytes were obtained from cerebral cortices of male/female 1-2-day-old Wistar rat pups, as previously described (Ramirez et al., 2019). Wild-type and SR-A knockout mice-derived cortical astrocyte cultures were prepared using the protocol of von Bernhardi and Eugenin (Von Bernhardi and Eugenin, 2004). Astrocyte cultures were maintained until the third week and were routinely close to 95% purity, as assessed by immunostaining with anti-GFAP monoclonal antibody (Millipore, MA, USA). Lack of SR-A in cultured astrocytes derived from SR-A knockout mice was previously demonstrated (Cornejo et al., 2018).

2.3. Treatments

After trypsinization from culture flasks, astrocytes were seeded on plaques or glass coverslips depending on the experimental assay and were treated with 0.1 μ M LY379268 (mGlu3R agonist; Tocris Biosciences, Bristol, UK) or with 1–10 nM recombinant sAPP α (S9564 Sigma-Aldrich, Buenos Aires, Argentina) in DMEM supplemented with 2 mM L-glutamine, 50 mg/mL streptomycin and 50 U penicillin without serum for 3, or 24 h as appropriate.

2.4. Glial conditioned media (GCM)

Were obtained as previously described (Durand et al., 2017). Briefly, 1×10^6 astrocytes were incubated with LY379268 or vehicle for 3 h, conditioned medium was collected and either used as complete GCM or subjected to immunoprecipitation for sAPP α using anti-sAPP α (2B3) antibody (Immuno-Biological Laboratories Co., Ltd. MN, USA) as we previously described (Durand et al., 2017). Supernatants were used as sAPP α -immunodepleted GCM. Naïve astrocytes were then exposed to either complete control GCM, complete LY GCM, or sAPP α -immunodepleted LY GCM for 24 h. A β uptake was determined after a further 24 h of treatment with fluorescent A β .

2.5. A β uptake quantification

After treatments, astrocytes (7×10^3 cells per glass coverslip) were incubated with 5 μ M A β ₂₅₋₃₅ HiLyte-fluorTM Alexa⁴⁸⁸ (Anaspec Inc. CA, USA) for 24 h. Cells were fixed with 4% paraformaldehyde and identified by GFAP staining (Durand et al., 2017). Nuclei were counterstained with DAPI. Uptake was analyzed under a fluorescence microscope (Axiophot, Carl Zeiss, Jena, Germany) and expressed as either the percentage of A β -positive astrocytes of total counted astrocytes (% phagocytic cells) or the fluorescence intensity per phagocytic cell calculated using ImageJ software (NIH). To determine the percentage of phagocytic cells, an average of 500 cells was counted per group for each independent experiment. To quantify fluorescence intensity per phagocytic cell, all phagocytic cells found in 15–20 random fields were analyzed per group for each independent experiment.

Residual fluorescence in culture supernatants was also quantified using a Synergy HT microplate reader (Biotek, USA).

2.6. A β uptake inhibition assay

Astrocytes were treated with LY379268 or sAPP α for 24 h, then either the medium was replaced by fresh medium containing 5 μ M A β ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ or A β ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ was added directly to the supernatants containing the residual drugs. A β uptake was assayed after 24 h.

In another set of experiments, astrocytes were treated with vehicle or 1 nM sAPP α for 24 h followed by medium replacement by either 5 μ M fluor-A β or a mix containing 5 μ M fluor-A β and 1 nM sAPP α (previously incubated at 37 °C for 1 h). After 24 h, A β uptake was determined. Experimental groups were named as follows:

Vehicle + fluor-A β : control group;

sAPP α + fluor-A β : sAPP α group;

sAPP α + fluor-A β / sAPP α mix: sAPP α + sAPP α /A β group;

Vehicle + fluor-A β / sAPP α mix: DMEM + sAPP α /A β group

Also, astrocytes were treated with vehicle for 24 h, then medium was replaced by anti-SR-A antibody (0.8 μ g/ml) to block SR-A for 1 h, and then replaced by the fluor-A β /sAPP α mix (previously incubated at 37 °C for 1 h) ('DMEM + anti-SR-A + sAPP α /A β ' group).

2.7. Reverse transcription and real-time PCR

Reverse transcription was performed from total astrocyte RNA as previously described (Durand et al., 2017). Rat SR-A1 (NM_001191939.1), insulin-degrading enzyme (IDE, NM_013159.1) and neprilysin (NEP, NM_012608.2) genes were analyzed using the following PCR primer sets: SR-A forward 5'-CAG ACT CAA AAG CTG GCC TTC T-3', SR-A reverse 5'-CGG TTG CCA TGC TGA AGT T-3', IDE forward 5'-CGC ACA GAG CAG TGG TAT GG-3', IDE reverse 5'-GAA TGA CGT CCT CTG GGA TAG C-3', NEP forward 5'-TGC AGC CCC CAT TCT TTA GT-3', NEP reverse 5'-CGA TGC CCC CAT AGT TCA AT-3'.

HPRT was used as endogenous control (HPRT forward 5'-CTCATG GACTGATATGGACAGGAC-3', HPRT reverse 5'-GCAGGTCAGCAAAG AACTTATAGCC-3'). PCR amplifications were done in a StepOne_ Real-Time PCR System (Applied Biosystems, Life Technologies Co, CA, USA) and PCR reactions were set up to a final volume of 16 μ L containing 4 μ L of cDNA, 200 nM primers (for SR-A/HPRT) or 250 nM primers (for IDE/HPRT) or 350 nM (for NEP), and SYBR Select Master Mix (Applied Biosystem). PCR conditions were UDG activation at 50 °C for 2 min and denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR product specificity was verified by a melting curve analysis. No-RT controls omitted the reverse transcriptase enzyme, and no-template controls were performed by addition of nuclease-free water instead of cDNA. Levels of SR-A, IDE, and NEP expression were normalized to the endogenous control gene HPRT and analyzed with Step-One Software (Applied Biosystems) using the comparative $\Delta\Delta$ Ct method. Data were reported as RQ mean of 2–4 biological replicates (with 3 technical replicates each) \pm SE.

2.8. Western blot

Astrocyte cultures (1×10^6 cells) were treated with LY379268 or recombinant sAPP α for 24 h. Total protein extracts were obtained after lysis with 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, and protease inhibitors (2 mM PMSF and protein inhibitor cocktail Sigma-Aldrich) followed by sonication and centrifugation at 12,000 rpm for 30 min. Protein concentration in supernatants was determined by the Bradford method (BioRad Laboratories, CA, USA) using bovine serum albumin as standard. Fifty micrograms of protein were size-fractionated in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were electrotransferred to a polyvinylidene difluoride membrane and blots were blocked in 1% or 5% nonfat dry milk-TBS-0.1% Tween 20 for 1 h and incubated overnight at 4 °C with 1:100 anti-SR-A (sc-1661396 Santa Cruz Biotechnology, TX, USA) or 1:200 anti- β -actin (sc-47778 Santa Cruz Biotechnology) in 1% or 5% milk-TBS-0.1% Tween 20. After 1 h incubation with the corresponding biotinylated secondary antibodies (Millipore Co., MA, USA) and 1 h incubation with Streptavidin-peroxidase (Thermo Fisher, Buenos Aires, Argentina), immunoreactivity was detected by enhanced chemiluminescence (BioLumina, Productos Bio-Lógicos, Buenos Aires, Argentina). SR-A and β -actin expression were analyzed on the same membrane after stripping. Bands were analyzed using SCION Image software. Results were normalized to the internal control and expressed as arbitrary units relative to the corresponding controls.

2.9. Co-immunoprecipitation assays

Total protein extracts (100 μ g) from cultured astrocytes were diluted in lysis buffer (50 mM Tris-HCl pH 7.5, 140 mM NaCl, 0.1% NP-40, 1 mM PMSF, 200 μ M Na₃VO₄, 1 mM NaF, and protease inhibitors cocktail). After preclearing with protein A/G agarose at 4 °C for 2 h to reduce non-specific adsorption, proteins were incubated with 1–2 μ g anti-SR-A (sc-1661396 Santa Cruz Biotechnology) at 4 °C with continuous shaking overnight. Immune complexes were precipitated using 20 μ L protein A/G agarose/tube at 4 °C for 2 h. After washing the pellet 3 times with washing buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% NP-40, 1 mM PMSF, 200 μ M Na₃VO₄, 1 mM NaF, and protease inhibitors cocktail), pellets were resuspended in 25 μ L sample buffer containing β -mercaptoethanol. coIP proteins were loaded onto 8% SDS-PAGE and transferred to PVDF membranes, which were blocked in 5% or 1% nonfat dry milk-TBS-0.1% Tween 20 and incubated overnight at 4 °C with 1:50 anti-sAPP α (#11088, clone 2B3, Immuno-Biological Laboratories Co., Ltd.) or 1:100 anti-SR-A (Santa Cruz Biotechnology) in 5% or 1% milk-TBS-0.1% Tween 20, respectively. After 1 h incubation with the biotinylated secondary antibody (Millipore Co.) and 1 h incubation with Streptavidin-peroxidase (Thermo Fisher), immunoreactivity was detected by enhanced chemiluminescence (BioLumina, Productos Bio-Lógicos). sAPP α and SR-A expression was analyzed on the same membrane after stripping. In parallel, total protein extracts (50 μ g) were loaded onto the same SDS-PAGE in order to confirm the presence of SR-A in each sample (input). Since sAPP α was not detected in total protein extracts from astrocytes (input), we used 2 μ g recombinant sAPP α as a positive control for sAPP α detection. As a negative control, IP was performed using an irrelevant, same-isotype, and same-species antibody.

Since both SR-A and sAPP α antibodies were generated in mice, we detected in the Western blot two bands corresponding to mouse IgG aside from the specific bands for SR-A and sAPP α .

2.10. Immunofluorescence

Astrocytes (7×10^3 cells/coverslip) were fixed with 4% paraformaldehyde, blocked in PBS plus 10% donkey and goat serum, and incubated with primary antibodies diluted in PBS plus 1% donkey and goat serum overnight at 4 °C as following: 1:100 anti-SR-A (ab123946, Abcam, Cambridge, UK) and 1:25 anti-sAPP α (Immuno-Biological Laboratories). After washing, cells were labeled with donkey anti-mouse and goat anti-rabbit IgG conjugated with Cy3 or FITC and counterstained with DAPI. Images were obtained using a fluorescence microscope (AxioPhot, Carl Zeiss, Jena, Germany) at 400X magnification.

2.11. MTT assays

Astrocytes (4×10^4 cells/well) were treated with either fibrillar (A β ₂₅₋₃₅ incubated at 37 °C for 3 days) or non-fibrillar A β ₂₅₋₃₅ (25 μ M, Sigma-Aldrich) in the presence or absence of recombinant sAPP α (1–10 nM) for 24 h. Metabolic activity of viable cells was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Durand et al., 2010).

3. Results

3.1. Astroglial SR-A levels increased in response to mGlu3R activation and sAPP α

Considering that mGlu3R activation increases A β clearance by astrocytes (Durand et al., 2017), and SR-A plays a main role in the decline in A β removal during AD (Cornejo et al., 2018), we aimed to investigate SR-A involvement in the aforementioned mGlu3R effect. First, we assessed whether SR-A levels are modified after mGlu3R activation. Both

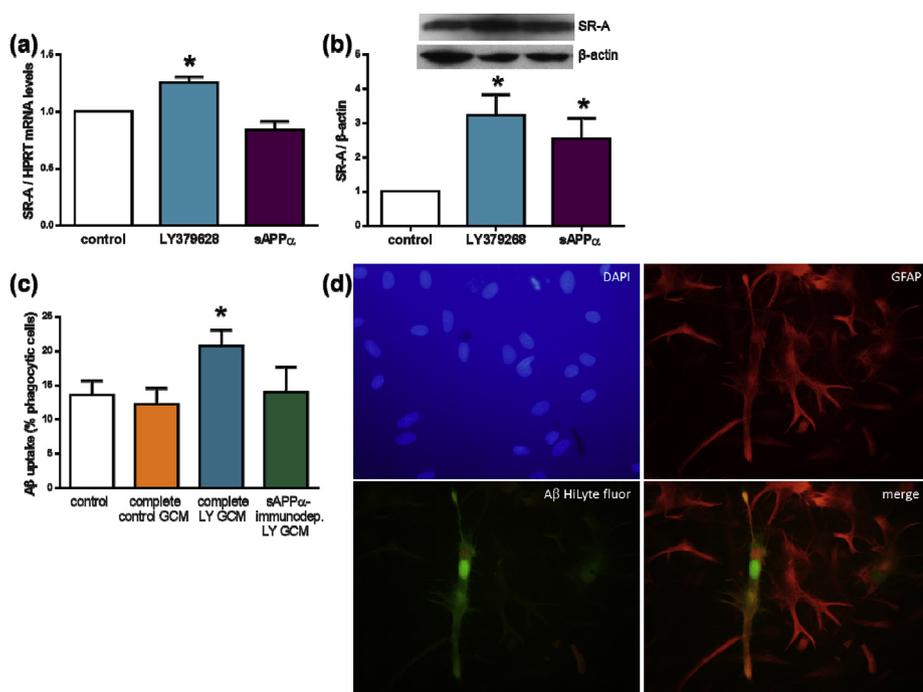


Fig. 1. sAPP α involvement in A β clearance by astrocytes. Rat primary astrocyte cultures were incubated with the mGlu3R agonist LY379268 (0.1 μ M) or recombinant sAPP α (1 nM) for 24 h and mRNA (a) or protein (b) levels of the class-A scavenger receptor (SR-A) were determined by real-time RT-PCR or Western blot, respectively. Data were expressed as mean \pm SE of RQ (a) or of arbitrary units relative to the control group (b) from 4 independent experiments. * $p < 0.05$ versus control group (Student's *t*-test). (c) Astrocytes were treated with vehicle or LY379268 for 3 h, then glial conditioned medium (GCM) was collected and half volume subjected to sAPP α immunodepletion using an anti-sAPP α antibody and protein A/G agarose. Naïve astrocytes were incubated for 24 h with complete control GCM, complete LY GCM, or sAPP α -immunodepleted LY GCM, followed by a further 24 h treatment with 5 μ M A β ₂₅₋₃₅ HiLyte fluor⁴⁸⁸. A β uptake was determined under a fluorescence microscope (Zeiss 40x objective) and expressed as the percentage of phagocytic cells of total counted cells. Data were shown as mean percentage \pm SE from 4 independent experiments. * $p < 0.05$ versus complete control GCM group (One way ANOVA followed by Bonferroni's test). A representative image is shown in (d).

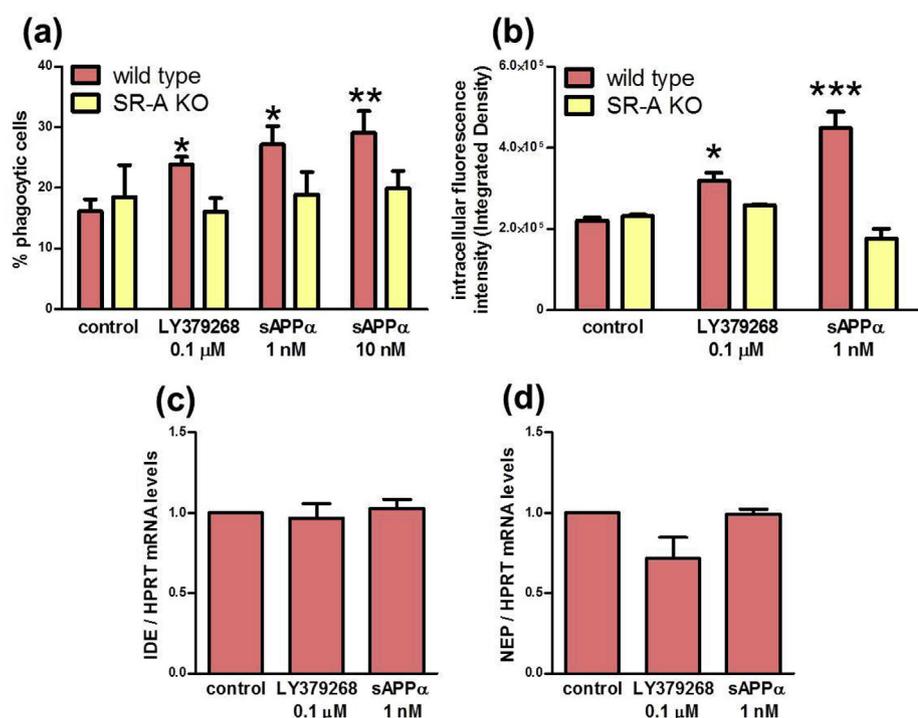


Fig. 2. mGlu3R- and sAPP α -induced A β uptake by astrocytes depend on SR-A. Primary astrocyte cultures derived from wild-type (pink bars) or SR-A knockout (yellow bars) mice were incubated with 0.1 μ M LY379268 or 1–10 nM recombinant sAPP α for 24 h. Then medium was replaced by fresh medium containing 5 μ M A β ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ for a further 24 h. A β uptake was determined by fluorescent microscopy and expressed as the percentage of phagocytic cells of total counted cells (a). Intracellular fluorescence intensity per phagocytic cell was quantified using ImageJ (b). Bars represent the mean \pm SE from 3 to 4 independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control of same genotype, Two way ANOVA followed by Bonferroni's test). (c and d) Rat primary astrocyte cultures were incubated with 0.1 μ M LY379268 or 1 nM recombinant sAPP α for 24 h and mRNA levels of IDE (c) and NEP (d) were determined by real-time RT-PCR. Data were expressed as RQ mean \pm SE from 2 independent experiments.

mRNA and protein levels of SR-A increased after astrocyte treatment with the mGlu3R agonist LY379268 (0.1 μ M, Fig. 1a and b).

Since sAPP α has been linked to enhanced SR-A-mediated A β uptake by microglial cells (Habib et al., 2017), we next studied the effect of recombinant sAPP α on astroglial SR-A levels. Although mRNA levels remained unchanged (Fig. 1a), SR-A protein expression was significantly increased by 1 nM sAPP α (Fig. 1b).

3.2. Release of sAPP α after mGlu3R activation mediated A β clearance

sAPP α involvement in mGlu3R-induced A β uptake by astrocytes was further demonstrated by treating naïve astrocytes with conditioned

medium (GCM) from astrocytes treated with LY379268 (LY-GCM). Complete LY-GCM increased the uptake of fluorescently-labeled A β ₂₅₋₃₅ by naïve astrocytes by 60% compared to complete control-GCM (Fig. 1c). However, when LY-GCM was immunodepleted of sAPP α , the aforementioned stimulatory effect was lost (Fig. 1c), evidencing an autocrine or paracrine role of sAPP α in the mGlu3R-induced A β uptake by astrocytes. Although an effect of remnant LY379268 in the GCM on naïve astrocytes could be possible in these experiments, the fact that sAPP α depletion from LY-GCM completely inhibited the LY-GCM-induced A β uptake allows us to discard this possibility. Moreover, we previously reported that LY-GCM subjected to IP in absence of anti-sAPP α antibody did not modify the effect of complete LY-GCM on

neuron viability (Durand et al., 2017), thereby discarding any technical artifact of immunoprecipitation in the observed biological effects.

As seen earlier for A β ₁₋₄₂ (Barucker et al., 2014; Dominguez-Prieto et al., 2018), A β ₂₅₋₃₅ was localized post engulfment in both astrocyte cytoplasm and nucleus (Fig. 1d).

3.3. SR-A involvement in mGlu3R- and sAPP α -induced A β uptake

To define the participation of SR-A in mGlu3R-induced A β uptake, fluorescently-labeled A β ₂₅₋₃₅ phagocytosis was measured in primary cultured astrocytes from wild-type and SR-A knockout mice. After treating astrocytes with LY379628 (0.1 μ M) or sAPP α (1 or 10 nM), medium was replaced by fresh medium containing 5 μ M A β ₂₅₋₃₅ HiLyte fluor⁴⁸⁸. After 24 h, A β uptake was determined by fluorescent microscopy and expressed as the percentage of phagocytic cells in the total counted cells. Whereas all treatments increased A β phagocytosis in wild-type astrocytes, none affected A β uptake in SR-A-deficient astrocytes (Fig. 2a). This result was also corroborated by measuring the A β remaining in culture media (data not shown).

Quantification of intracellular fluorescence in phagocytic cells revealed increased fluorescence intensity in LY379268 and sAPP α groups for wild-type astrocytes (Fig. 2b). As expected, none of the treatments affected intracellular fluorescence levels in SR-A-deficient astrocytes (Fig. 2b). Therefore, LY379268- and sAPP α -induced increase of A β accumulation in astrocytes required the presence of SR-A, since it was abolished in SR-A deficient cells, indicating that this process was most likely mediated by SR-A.

We also measured expression of the two most important A β degrading enzymes in wild-type astrocytes: insulin-degrading enzyme (IDE) and neprilysin (NEP). Surprisingly, neither LY379268 nor sAPP α modified mRNA levels of these catalytic enzymes (Fig. 2c and d). Therefore, mGlu3R/sAPP α pathway might promote A β uptake and accumulation into astrocytes rather than its intracellular degradation.

3.4. The presence of residual sAPP α in supernatants inhibited A β uptake

An interesting and surprising finding emerged when we compared results from phagocytosis assays performed by two different approaches: first, as described in Fig. 2, after treating astrocytes with LY379628 or sAPP α , medium was completely replaced by fresh medium containing fluorescent A β , and A β uptake was determined 24 h later (Fig. 3a). Alternatively, after treating astrocytes with LY379628 or sAPP α , fluorescent A β was added to the culture medium containing incubation drugs (Fig. 3b). In both cases, the control group was set as 100% and data relativized to 100%.

The stimulatory effect of sAPP α on A β uptake observed when the medium was replaced (Fig. 3a) was abolished when A β was added to the incubation medium with drugs (Fig. 3b). The stimulatory effect of LY379268 when medium was replaced (an approximately 25% increase in phagocytic cells, Fig. 3a) was lowered to around 10% when A β was added to the incubation medium (Fig. 3b).

Considering that sAPP α can interact with SR-A (Santiago-Garcia et al., 2001) as well as with A β (Darlington et al., 2015), one possible explanation for our observations could be that, in the second case, residual sAPP α present in the medium (both recombinant and endogenous sAPP α released in response to LY379268) was binding to A β and sequestering it, thereby inhibiting A β interaction with SR-A, a scenario schematically represented in Fig. 3c.

In order to address this issue, we designed an alternative experimental scheme (Fig. 4a): astrocytes were incubated with vehicle (1st and 4th column) or sAPP α (2nd and 3rd column) for 24 h; then, supernatant was replaced by fluorescent A β (1st and 2nd column) or by a mixture of sAPP α + fluorescent A β previously incubated for 1 h at 37 °C to allow for their interaction (3rd and 4th column), as described by Darlington et al. (2015). After a further 24 h, A β uptake was analyzed. Our results show that A β /sAPP α complexes (obtained after *in*

vitro heterodimerization) inhibited A β uptake in astrocytes pre-treated with sAPP α (Fig. 4a and b, 3rd column) versus the sAPP α group (Fig. 4a and b, 2nd column). However, when astrocytes were not pre-treated with sAPP α (therefore presumably not saturated SR-A) the mentioned inhibitory effect of A β /sAPP α complexes was reversed (Fig. 4a and b, 4th column). On the contrary, A β /sAPP α complexes were unable to change A β uptake when SR-A was blocked with an anti-SR-A antibody (Fig. 4a and b, 5th column). Altogether, these results indicate that free and SR-A-associated sAPP α may compete for A β binding, further suggesting that sAPP α binding to SR-A may enhance SR-A dependent A β uptake.

3.5. sAPP α /SR-A dimerization

Interaction between sAPP α and SR-A at the astrocyte's membrane was studied. Co-immunoprecipitation (IP) assays from protein lysates were performed using anti-SR-A antibody for IP followed by immunoblot (IB) for sAPP α (Fig. 5a). We detected a 75 kDa-band corresponding to sAPP α in SR-A-precipitated samples (red arrowhead), which evidences direct interaction between these proteins. Since sAPP α is undetectable in the input sample (cellular lysates), recombinant sAPP α was run as a positive control. As an IP control, the same membrane was stripped and assayed by immunoblot for SR-A (Fig. 5b). The specific band for SR-A (white arrowhead) was detected in immunoprecipitated samples as well as in the input. In the negative control the same unspecific band was observed in the two IB.

We also detected SR-A and sAPP α in non-permeabilized astrocytes by immunofluorescence, indicating that these proteins are associated with the plasma membrane (Fig. 5c–e). Using double immunofluorescence labeling, we observed that SR-A and sAPP α were closely localized in the astrocyte plasma membrane (Fig. 5c and d). A punctuate staining pattern seen for sAPP α coincides with the SR-A staining pattern in several regions of the plasma membrane of most cells. This circumscribed staining pattern is coherent with previously reported localization of sAPP α in membrane microdomains or rafts in other cell types (Hoffmann et al., 1999; Tikkanen et al., 2002).

3.6. Astrocyte viability was not affected by sAPP α

To investigate whether sAPP α can influence other cellular processes, beyond its particular autocrine/paracrine role in A β uptake by astrocytes, we analyzed sAPP α effect on astrocyte viability after A β exposure. Cell viability was significantly decreased by 25 μ M A β ₂₅₋₃₅ (Fig. 6a and b). The detrimental effect of A β on cell viability was independent of its aggregation status and was not modified by either 1 or 10 nM sAPP α (Fig. 6a and b). sAPP α had no effect *per se* on astroglial viability (Fig. 6a and b). Therefore, autocrine/paracrine action of sAPP α in astrocytes appears to affect A β elimination, but does not protect cells from A β toxicity, which differs from our previous results for neurons (Durand et al., 2017).

4. Discussion

In the field of AD, much interest is focused on A β toxicity, and most proposed therapies aim to mitigate A β production. However, it is likely that parallel deficiency in non-amyloidogenic cleavage (and consequently, ineffective sAPP α release) has a major impact on AD onset/progression. Also, astrocyte neuroprotective functions may have major therapeutic relevance for AD. In fact, glial cell dysregulation has been suggested as a possible cause of AD (Cornejo et al., 2018; Cai et al., 2017). In the present study, we demonstrated that sAPP α produced by astrocytes in response to mGlu3R activation could act in a paracrine or autocrine fashion to stimulate A β uptake by astrocytes. We also showed that sAPP α - and mGlu3R-induced A β internalization by astrocytes depends on SR-A participation; we further suggest that sAPP α might dimerize with SR-A to enhance A β uptake. These results complement our

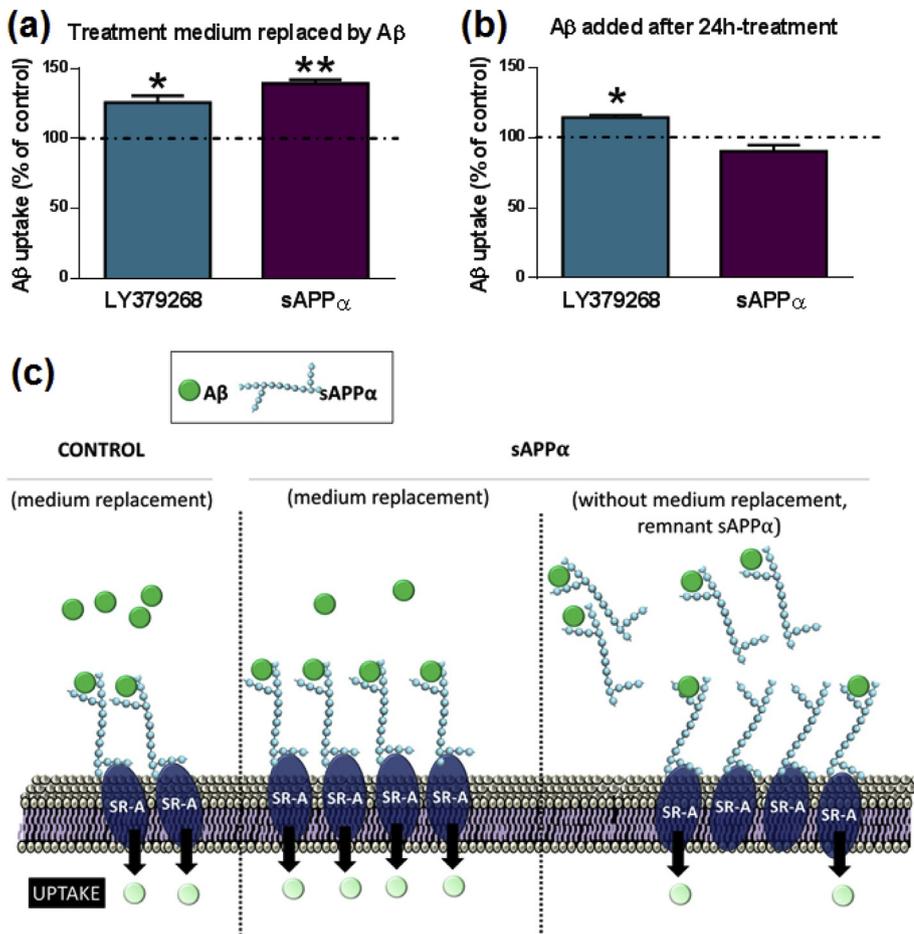


Fig. 3. Residual sAPP α in supernatants inhibits A β uptake by astrocytes. Astrocytes were treated with vehicle, LY379268, or sAPP α for 24 h and then either the medium was replaced by fresh medium containing 5 μ M A β ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ (a) or else the A β ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ was added directly to the culture media containing the drugs (b). After 24 h, A β uptake was determined and the percentage of phagocytic cells was expressed relative to the corresponding control group, which was set as 100%. *p < 0.05, **p < 0.01 versus control group (Student's *t*-test, n = 3 independent experiments). A hypothesis to explain the results is represented in (c): if SR-A at the cell membrane binds sAPP α and this, in turn, binds to A β , then the presence of free sAPP α in the culture media would compete with SR-A-bound sAPP α for A β binding, thereby avoiding A β clearance.

previous work showing that astrocyte-derived sAPP α released after mGlu3R activation prevents hippocampal neuron death induced by A β (Durand et al., 2017), and contribute to consolidate the mGlu3R/sAPP α pathway as a relevant therapeutic target for AD.

Whereas A β production is accelerated in many familial forms of early-onset AD, increasing evidence indicates that impaired clearance of A β is more evident in late-onset AD (Liu et al., 2017). Despite a long delay in recognizing astrocyte phagocytic potential compared to microglia or macrophages, the participation of plaque-associated astrocytes in A β elimination from AD brains is now well known. Unlike microglia, a fundamental singularity of astrocytes in the A β degradation process is their ability to internalize and degrade A β without requiring pre- or co-stimulation by cytokines (Pihlaja et al., 2011). Several surface mediators have been suggested as putative A β -binding partners in astrocytes, including SR. However, divergent evidence has been presented on SR expression in glial cells. Whether studies are made using newborn or adult glial cells, plaque-associated (or not) cells, under certain pathophysiological conditions, or in knockout animal models are all factors that can affect the expression pattern and/or activity of SR in astrocytes and microglia, or even modify the identity of the SR involved in A β elimination. Frenkel et al. (Frenkel et al., 2013) showed that SR-A deficiency clearly impairs A β clearance and reduces NEP and IDE expression in the APP/PS1 AD model. Concordantly, SR-A mediates oligomeric A β internalization and further lysosomal degradation by cathepsin B in microglia (Yang et al., 2011). A reduction in hippocampal SR-A expression with age has also been reported, appearing earlier in APP/PS1 mice than in wild-type animals (Cornejo et al., 2018). These changes were attributed mainly to altered expression of SR-A in microglial cells rather than in astrocytes and were accompanied by impaired microglial phagocytic activity, impaired

working memory, and higher mortality rate (Cornejo et al., 2018). Iram et al. (Iram et al., 2016) demonstrated that adult astrocytes isolated from old 5xFAD mice show impaired A β clearance and that incubation of A β with C1q enhances phagocytic activity, which was suggested to depend on SR-B1, not SR-A. In contrast, our present results show that both mGlu3R activation as well as recombinant sAPP α induced SR-A protein expression and SR-A-dependent A β uptake. However, these findings refer to mGlu3R- and/or sAPP α -induced A β uptake by post-natal astrocytes, whereas Iram's experiments reflect changes in old, AD mice-derived astrocytes. Considering the decline in SR-A expression with age, it is likely that a compensatory increase in SR-B1 levels accounts for C1q-stimulated A β uptake, as suggested by Husemann et al. (Husemann et al., 2002). However, our results do not support a redundancy mechanism scenario since SR-A depletion completely blocked mGlu3R- and sAPP α -induced A β phagocytosis. Our present results further show that SR-A-deficient astrocytes retained their basal phagocytic capacity although they lacked response to LY379268 or sAPP α stimuli, a finding that might tally with reported lack of SR-A involvement in basal A β phagocytosis. Therefore, we are hereby characterizing a pathway that might be specific for mGlu3R/sAPP α -stimulated A β clearance.

The therapeutic potential of sAPP α has been highlighted since the discovery of its neurotrophic and neuroprotective actions on neural cells (reviewed in (Habib et al., 2017)). Aside from its direct neurotrophic action, sAPP α can allosterically bind and inhibit β -secretase, leading to reduced A β synthesis (Obregon et al., 2012; Peters-Libeu et al., 2015). sAPP α also reduces tau hyperphosphorylation and GSK3 β activity in neural cells and in the APP/PS1 model (Deng et al., 2015), and can improve cognition (reviewed in (Habib et al., 2017)). sAPP α enhances SR-A-mediated A β phagocytosis by microglia and monocytes

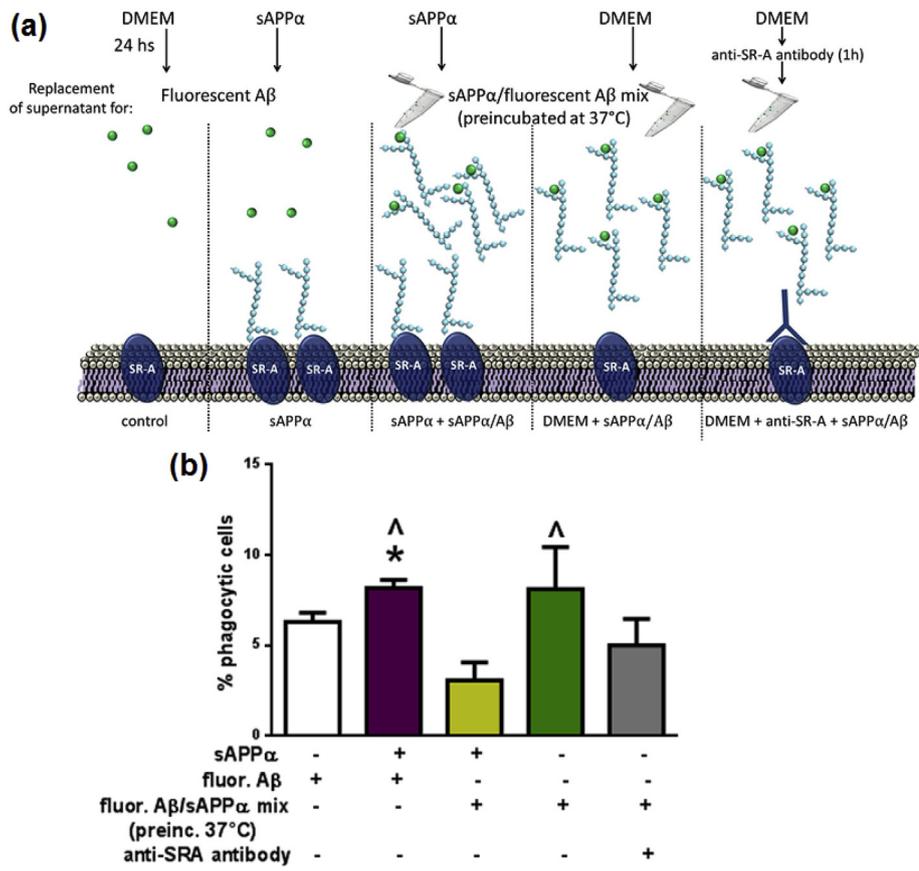


Fig. 4. sAPPα interacts with Aβ and SR-A to enhance Aβ uptake. (a) Schematic representation of the experimental design:

'Control' group: astrocytes were treated with vehicle for 24 h. Then, medium was replaced by Aβ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ for a further 24 h; first column in (b)

'sAPPα' group: astrocytes were treated with sAPPα for 24 h. Then, medium was replaced by Aβ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ for a further 24 h; second column in (b)

'sAPPα + sAPPα/Aβ' group: astrocytes were treated with sAPPα for 24 h. Then, medium was replaced by fresh medium containing Aβ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ and sAPPα (previously incubated at 37 °C for 1 h) for a further 24 h; third column in (b)

'DMEM + sAPPα/Aβ' group: astrocytes were treated with vehicle for 24 h. Then, medium was replaced by fresh medium containing Aβ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ and sAPPα (previously incubated at 37 °C for 1 h) for a further 24 h; fourth column in (b)

'DMEM + anti-SR-A + sAPPα/Aβ' group: astrocytes were treated with vehicle for 24 h. Then, medium was replaced by anti-SR-A antibody (0.8 μg/ml) to block SR-A for 1 h, then medium was replaced by fresh medium containing Aβ₂₅₋₃₅ HiLyte fluor⁴⁸⁸ and sAPPα (previously incubated at 37 °C for 1 h) for a further 24 h; fifth column in (b).

In (b) Aβ uptake was determined and expressed as the percentage of phagocytic cells. Bars represent the mean percentage ± SE from 4 independent experiments. (*p < 0.05 versus control group; ^p < 0.05 versus 'sAPPα + sAPPα/Aβ' group, One way ANOVA followed by Bonferroni's test).

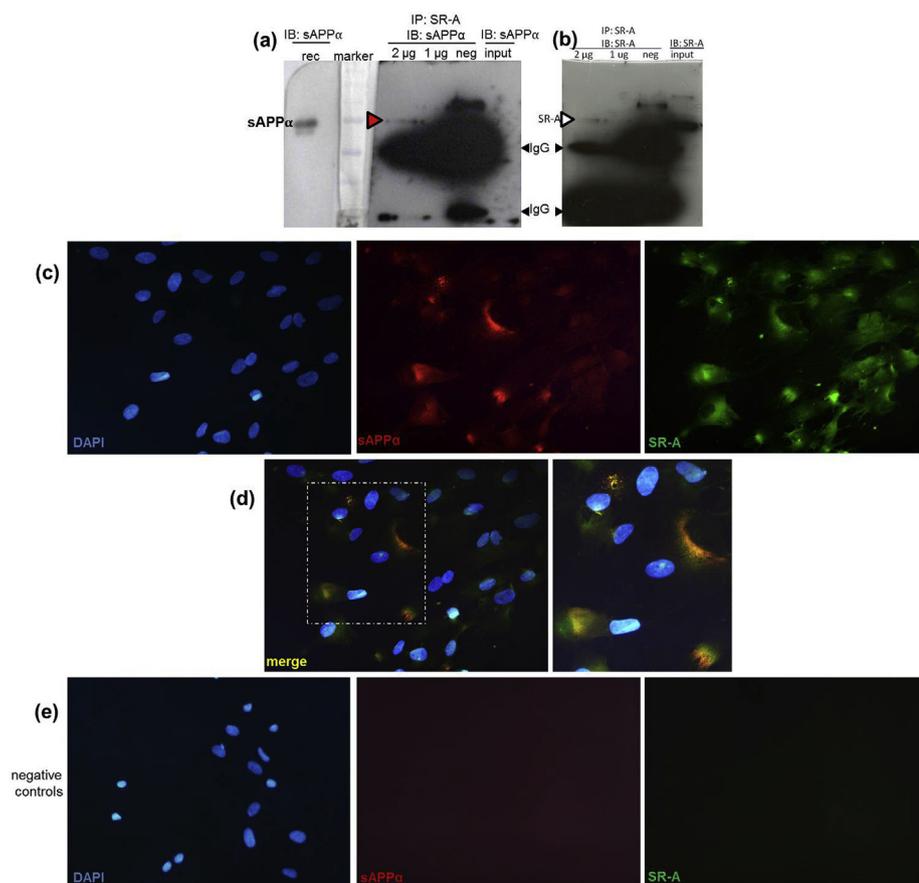


Fig. 5. sAPPα-SR-A binding. Protein lysates (100 μg) from astrocytes were immunoprecipitated (IP) using 1 or 2 μg of anti-SR-A antibody and protein A/G agarose and analyzed by immunoblot (IB) using anti-sAPPα antibody (a) or anti-SR-A antibody (b). Positive bands are indicated by red (sAPPα) or white (SR-A) arrowheads. Neg.: negative control (lysates immunoprecipitated with 2 μg of an irrelevant antibody; the same unspecific band was detected in the two blots). Input: complete protein lysate without IP; sAPPα was undetectable in cell lysates. As positive control, 2 μg of recombinant (rec) sAPPα were analyzed by Western blot. sAPPα and SR-A expression was analyzed on the same membrane after stripping. Since both SR-A and sAPPα antibodies were generated in mice, bands corresponding to IgG's are present in the IB's. The same result was obtained in 3 independent experiments.

(c) Immunofluorescence for SR-A and sAPPα in primary cultured astrocytes was performed without permeabilizing membranes in order to detect membrane-associated peptides (Zeiss 40x objective). (d) Merging was done using Adobe Photoshop CS. Higher magnification of inset is shown on the right. (e) Negative control: immunofluorescence in absence of primary antibodies.

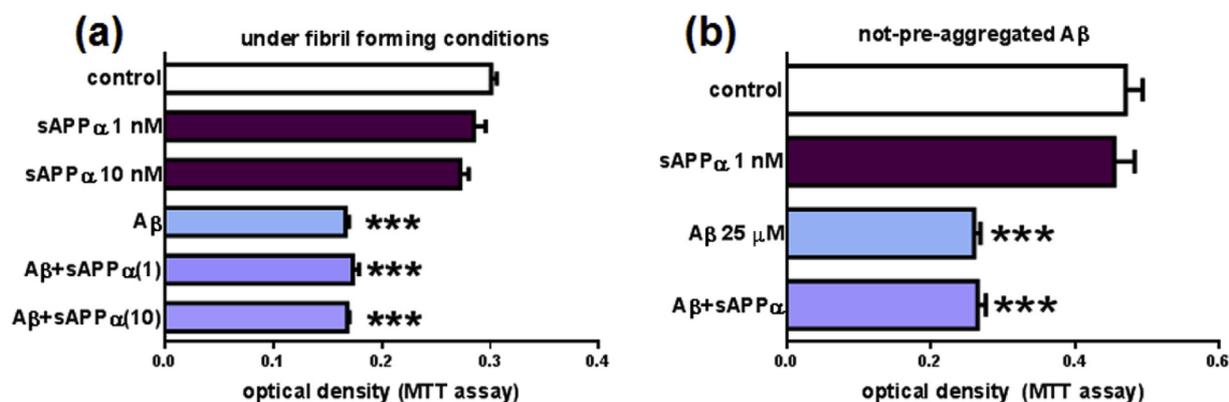


Fig. 6. The detrimental effect of A β on astrocyte viability is not prevented by sAPP α . Astrocytes were incubated with 25 μ M A β ₂₅₋₃₅ either as fibrils (pre-aggregated A β at 37 $^{\circ}$ C for 3 days) (a) or without pre-aggregation (b) in the presence or absence of sAPP α (1–10 nM) for 24 h. Cell viability was determined by the MTT assay. *** p < 0.001 versus control group (One-way ANOVA followed by Bonferroni's test, n = 8 determinations from one experiment representative of two independent ones).

(Darlington et al., 2015), and sAPP α overexpression in APP/PS1 Δ E9 mice rescued structural, electrophysiological, and behavioral deficits in these mice through microglial activation, and increased A β clearance (Fol et al., 2016). However, although we know that astrocytes can also secrete sAPP α , which then enhances neuron survival (Habib et al., 2017; Durand et al., 2017), autocrine or paracrine effects of this peptide on astroglial cells have not been exhaustively explored. We show here for the first time that sAPP α promotes A β clearance by cultured astrocytes. Moreover, sAPP α release by astrocytes after mGlu3R stimulation is required for mGlu3R-induced astroglial A β uptake, since sAPP α -depleted conditioned medium from LY-treated astrocytes did not induce A β uptake by naïve astrocytes, as did complete LY-GCM. This result was corroborated by using recombinant sAPP α , which was also able to improve A β uptake by both rat and mouse astrocytes. After this uptake, we observed an accumulation of A β within astrocytes, indicated by increased intracellular fluorescence in the phagocytic cells, and found no changes in mRNA levels of the two most important glial A β degrading enzymes, IDE and NEP. Concordant with these observations, Söllvander, et al. (Söllvander et al., 2016) demonstrated that astrocytes do not degrade but instead store engulfed A β protofibrils, since protofibrils persist for a long time in the cytoplasm of cultured astrocytes.

Better understanding of sAPP α functions and binding partners is needed in order to comprehend its neuroprotective role. Interestingly, Hoffmann, et al. (Hoffmann et al., 1999) found that sAPP binding at the cell surface is saturable, specific, and dependent on cell differentiation, occurring in patches or microdomains. Signaling transduction after sAPP α binding to cell membranes should be mediated by a receptor protein that has yet to be univocally identified. Also, it is known that phagocytosis of A β by human astrocytes may depend on A β conformation as well as on its association with several amyloid-associated proteins (Nielsen et al., 2010). Our present results suggest that SR-A binds sAPP α and that this complex, in turn, binds A β to allow for A β clearance. Alternatively, sAPP α could associate with A β and then bind to SR-A to promote A β phagocytosis. However, sAPP α /A β complexes might be unable to attach to the cell when SR-A are pre-occupied by sAPP α . We further demonstrated sAPP α -SR-A interaction by coIP assay as well as by double labeled immunofluorescence, which revealed physical interaction between these proteins. Regarding immunofluorescence, despite ubiquitous APP expression, we were still able to detect sAPP α specifically after using clone 2B3 sAPP α antibody, which does not bind full length APP or sAPP β , as stated by the manufacturer. Altogether, our data support the hypothesis that sAPP α functions as a carrier that reinforces binding of A β to SR-A, thereby enhancing A β attachment and uptake by astrocytes. Previous evidence agrees with this hypothesis. A β is able to bind APP-derived peptides (Darlington

et al., 2015; Manocha et al., 2016; Verdier and Penke, 2004). All SR-A ligands are polyanions, and sAPP α has a negatively charged region thought to be responsible for binding to SR-A (Santiago-Garcia et al., 2001). Providing strong support for our findings, Darlington, et al. (Darlington et al., 2015) have shown that, whereas aged monocytes display ineffective phagocytosis of A β , pretreatment of aged monocytes with sAPP α restores their phagocytic potential. They also suggested that sAPP α could form a heterodimer with A β , thereby promoting binding of these heterodimers to monocyte scavenger receptors and enhancing A β clearance. Similar to our hypothesis, results from Zhang et al. (Zhang et al., 2014) indicate that heterodimerization between A β ₁₋₄₂ and peptide XD4 may be a prerequisite for enhanced internalization of A β ₁₋₄₂ by microglia and, further, that A β ₁₋₄₂ may be internalized as A β ₁₋₄₂-XD4 complex. Moreover, XD4-induced A β ₁₋₄₂ phagocytosis by microglia is dependent on SR-A.

Finally, we show here that A β ₂₅₋₃₅ (the shortest A β peptide displaying neurotoxicity (Kaneko et al., 2001)) exerted a toxic effect on cultured astrocytes, as previously reported (Dominguez-Prieto et al., 2018). This effect was independent of the state of aggregation of the peptide, since both fibrillar and non fibrillar A β ₂₅₋₃₅ induced similar levels of astrocyte death. In both cases, recombinant sAPP α had no effect on astrocyte viability, a finding that contrasts with the reported pro-survival effect of sAPP α on hippocampal neurons (Durand et al., 2017).

5. Conclusion

Growing knowledge of astroglial involvement in A β plaque clearance encourages research oriented towards restoring A β uptake mechanisms in astrocytes as promising therapy for AD. Although microglia is widely accepted as the star phagocytes in brain, microglial functions are also known to be impaired in aging/AD models. Therefore, conservation or stimulation of astroglial function to potentially compensate for the loss of microglial phagocytic action merits research efforts focused on clearing A β from AD brains. Our present results show that the mGlu3R/sAPP α pathway stimulates A β clearance by astrocytes in an SR-A-dependent fashion. We also suggest a double binding of sAPP α to SR-A and A β as the mechanism underlying A β clearance enhancement by mGlu3R and sAPP α . However, we must be cautious when extrapolating results obtained from neonatal astrocytes to adult/aged astrocytes, since developmental changes in SR expression are broadly reported.

Although sAPP α offers great therapeutic potential, it is too large to cross the BBB (Habib et al., 2017). Another predictable way to increase sAPP α levels in the brain would be to increase α -secretase activity, but this option involves severe secondary alterations in alternative α -

secretases substrates. Therefore, all evidence favors selective mGlu3R activation as a therapeutic target for AD, since its agonists can cross the BBB, while also exerting several neuroprotective actions and ensuring endogenous release of sAPP α from astrocytes.

Author contributions

Study conception and design: DD.
 Acquisition of data: DD, JT, MJR, JS, DR, RvB.
 Analysis and interpretation of data: DD, JT, MJR, LC, CC, RvB.
 Drafting of manuscript: DD, LC, CC.
 Critical revision: RvB, ML.

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Declarations of interest

None.

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