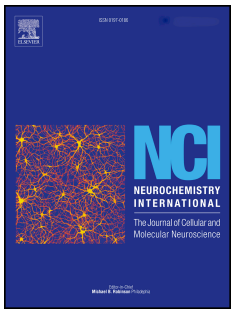

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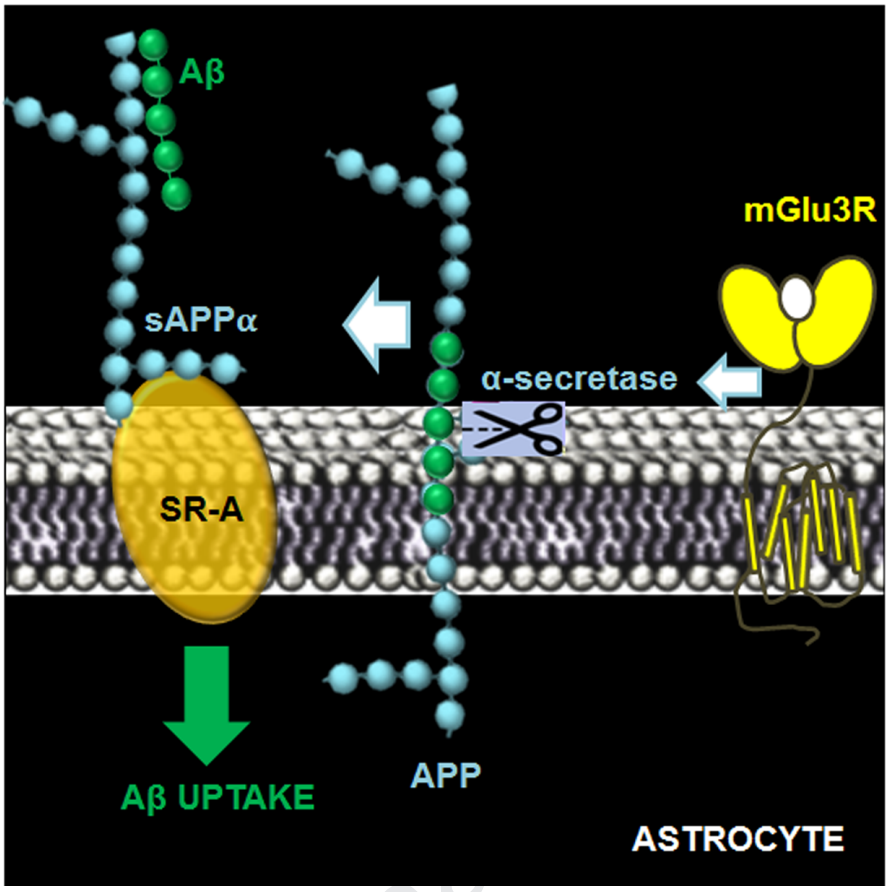

UNRAVELING THE β-AMYLOID CLEARANCE BY ASTROCYTES: INVOLVEMENT OF METABOTROPIC GLUTAMATE RECEPTOR 3, sAPPα, AND CLASS-A SCAVENGER RECEPTOR

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Declarations of interest: none.

Abstract

The mechanics of β-amyloid (Aβ) 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 (sAPPα) as well as on Aβ clearance; however, the mechanism underlying mGlu3R-induced Aβ uptake by astrocytes remains unclear. The present study shows that conditioned medium from mGlu3R-stimulated astrocytes increased Aβ uptake by naïve astrocytes through a mechanism dependent on sAPPα, since sAPPα depletion from conditioned medium inhibited Aβ phagocytosis. Concordantly, recombinant sAPPα also increased Aβ uptake. Since we show that both sAPPα 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 sAPPα-induced Aβ uptake by using astrocyte cultures derived from SR-A knockout mice. We found that the effects of LY379268 as well as sAPPα on Aβ uptake were abolished in SR-A-deficient astrocytes, indicating a major role for this scavenger receptor in LY379268- and sAPPα-stimulated Aβ clearance by astrocytes. We also show results of coimmunoprecipitation and functional assays offering evidence of possible heterotrimerization of sAPPα with Aβ and SR-A which could allow Aβ to enter the astrocyte. In conclusion the present paper describes a novel pathway for Aβ clearance by astrocytes involving sAPPα as an enhancer of SR-A-dependent Aβ phagocytosis.

Keywords: astrocytes, subtype 3 metabotropic glutamate receptor, sAPPα, β-amyloid clearance, class-A scavenger receptor, coimmunoprecipitation

Defined abbreviations:

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

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 [1, 2]. Neuron-glia interaction has been widely shown to promote neuroprotection, in part through mechanisms involving group II mGluR activation [3].

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 (Aβ) peptide from amyloid precursor protein (APP), the late-onset AD is thought to arise after failures in Aβ clearance mechanisms [4-6].

At the cell surface, APP undergoes proteolysis by α-secretases that cleave it between Lys_{687} and Leu_{688} thereby releasing a large, soluble ectodomain: sAPPα. Alternatively, APP can be cleaved by β-secretase to generate a fragment which -after further cleavage by γ-secretase- yields the Aβ peptide. We recently showed that mGlu3R activation by the agonist LY379268 promotes the non-amyloidogenic (or '*alpha'*) cleavage of APP in cultured astrocytes, leading to increased release of sAPPα [7], which has been demonstrated to exert neuroprotective action in several injury models and to improve cognition in AD and aging [8]. Furthermore, mGlu3R expression is diminished in hippocampal astrocytes from PDAPP-J20 mice [7], suggesting a role for this receptor in AD. In addition, our most recent results show that astrocyte-derived sAPPα protects hippocampal neurons from Aβ neurotoxicity [9]. Moreover, LY379268 is also able to increase Aβ uptake in both astrocytes and microglia, an effect that impacts positively on the survival of co-cultured neurons [9]. Nevertheless, how mGlu3R triggers Aβ engulfment by astrocytes is an unexplored research topic.

Glial cells are the main scavengers of the brain, and Aβ is primarily internalized through receptor-mediated endocytosis [10]. 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 Aβ uptake [10]. In microglia, we showed involvement of CD11b in the recognition/endocytosis of Aβ induced by Group II mGluR activation [9], whereas in astrocytes the underlying mechanism remains to be studied. Although we previously showed that LY379268 upregulates gene expression of SR CD36 in astrocytes [9], the literature points to class A SR (SR-A) as the central Aβ-interacting receptor in AD models. SR-A expression is reduced in the hippocampus of aged and APP/PS1 animals [11], 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 [11]. Consequently, these triple transgenic mice show accelerated Aβ accumulation, and pharmacological upregulation of SR-A expression on mononuclear phagocytes increases Aβ clearance [12]. Interestingly, an association between sAPPα and Aβ clearance has also been demonstrated: sAPPα can interact with several partners participating in the Aβ clearance mechanism, such as SR-A and LRP1 in microglia, neurons, peripheral monocytes, and endothelial cells from the blood-brain barrier [8]. sAPPα enhances SR-A-mediated phagocytosis of Aβ by microglia and monocytes [8], and upregulates Aβ-degrading enzymes in microglia from APP/PS1ΔE9 mice, thereby reducing Aβ plaques [13]. However, involvement of sAPPα in astroglial Aβ clearance was not yet revealed. Our present work shows molecular mechanisms involved in enhanced Aβ removal by the mGlu3R/sAPPα 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)[14] 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 genotypified 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 [15]. Wild-type and SR-A knockout mice-derived cortical astrocyte cultures were prepared using the protocol of von Bernhardi and Eugenín [16]. 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 [11].

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 μM LY379268 (mGlu3R agonist; Tocris Biosciences, Bristol, UK) or with 1-10 nM recombinant sAPPα (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 24h as appropriate.

2.4. Glial conditioned media (GCM) were obtained as previously described [9]. Briefly, $1x10^6$ astrocytes were incubated with LY379268 or vehicle for 3h, conditioned medium was collected and either used as complete GCM or subjected to immunoprecipitation for sAPPα using anti-sAPPα (2B3) antibody (Immuno-Biological Laboratories Co., Ltd. MN, USA) as we previously described [9]. Supernatants were used as sAPPα-immunodepleted GCM. Naïve astrocytes were then exposed to either complete control GCM, complete LY GCM, or sAPPα-immunodepleted LY GCM for 24h. Aβ uptake was determined after a further 24h of treatment with fluorescent Aβ.

2.5. Aβ uptake quantification. After treatments, astrocytes ($7x10^3$ cells per glass coverslip) were incubated with 5 μM $A\beta_{25-35}$ HiLyte-fluor™ $Alexa^{488}$ (Anaspec Inc. CA, USA) for 24 h. Cells were fixed with 4% paraformaldehyde and identified by GFAP staining [9]. Nuclei were counterstained with DAPI. Uptake was analyzed under a fluorescence microscope (Axiophot, Carl Zeiss, Jena, Germany) and expressed as either the percentage of Aβ-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. Aβ uptake inhibition assay. Astrocytes were treated with LY379268 or sAPPα for 24h, then either the medium was replaced by fresh medium containing 5μM $A\beta_{25-35}$ HiLyte fluor^{488} or $A\beta_{25-35}$ HiLyte fluor^{488} was added directly to the supernatants containing the residual drugs. Aβ uptake was assayed after 24h.
In another set of experiments, astrocytes were treated with vehicle or 1 nM sAPPα for 24h followed by medium replacement by either 5 μM fluor-Aβ or a mix containing 5 μM fluor-Aβ and 1 nM sAPPα (previously incubated at 37°C for 1h). After 24h, Aβ uptake was determined. Experimental groups were named as follows:
Vehicle + fluor-Aβ: control group;
sAPPα + fluor-Aβ: sAPPα group;
sAPPα + fluor-Aβ/ sAPPα mix: sAPPα + sAPPα/Aβ group;
Vehicle + fluor-Aβ/ sAPPα mix: DMEM + sAPPα/Aβ group.
Also, astrocytes were treated with vehicle for 24h, then medium was replaced by anti-SR-A antibody (0.8 μg/ml) to block SR-A for 1h, and then replaced by the fluor-Aβ/sAPPα mix (previously incubated at 37°C for 1h) ('DMEM + anti-SR-A + sAPPα/Aβ' group).

2.7. Reverse transcription and real-time PCR. Reverse transcription was performed from total astrocyte RNA as previously described [9]. Rat SR-A1 (NM_001191939.1), insulin-degrading enzyme (IDE, NM_013159.1) and neprilysine (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'-CTCATGGACTGATTATGGACAGGAC-3', HPRT reverse 5'-GCAGGTCAGCAAAGAACTTATAGCC-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 μL containing 4 μL 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 ΔΔCt method. Data were reported as RQ mean of 2-4 biological replicates (with 3 technical replicates each) ± SE.

2.8. Western blot. Astrocyte cultures ($1x10^6$ cells) were treated with LY379268 or recombinant sAPPα for 24h. 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)-polyacrilamide 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 1h 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 1h incubation with the corresponding biotinylated secondary antibodies (Millipore Co., MA, USA) and 1h incubation with Streptavidin-peroxidase (Thermo Fisher, Buenos Aires, Argentina), immunoreactivity was detected by enhanced chemiluminescence (Bio-Lumina, 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 µg) 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 µM Na_3VO_4, 1 mM NaF, and protease inhibitors cocktail). After preclearing with protein A/G agarose at 4°C for 2h to reduce non-specific adsorption, proteins were incubated with 1-2 µg anti-SR-A (sc-1661396 Santa Cruz Biotechnology) at 4°C with continuous shaking overnight. Immune complexes were precipitated using 20 µl protein A/G agarose/tube at 4°C for 2h. 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 µM Na_3VO_4, 1 mM NaF, and protease inhibitors cocktail), pellets were resuspended in 25 µl 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-sAPPα (#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 1h incubation with the biotinylated secondary antibody (Millipore Co.) and 1h incubation with Streptavidin-peroxidase (Thermo Fisher), immunoreactivity was detected by enhanced chemiluminescence (Bio-Lumina, Productos Bio-Lógicos). sAPPα and SR-A expression was analyzed on the same membrane after stripping. In parallel, total protein extracts (50 µg) were loaded onto the same SDS-PAGE in order to confirm the presence of SR-A in each sample (input). Since sAPPα was not detected in total protein extracts from astrocytes (input), we used 2 µg recombinant sAPPα as a positive control for sAPPα detection. As a negative control, IP was performed using an irrelevant, same-isotype, and same-species antibody.
Since both SR-A and sAPPα 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 sAPPα.

2.10. Immunofluorescence. Astrocytes ($7x10^3$ 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-sAPPα (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 ($4x10^4$ cells/well) were treated with either fibrillar ($A\beta_{25-35}$ incubated at 37°C for 3 days) or non-fibrillar $A\beta_{25-35}$ (25 µM, Sigma-Aldrich) in the presence or absence of recombinant sAPPα (1-10 nM) for 24h. Metabolic activity of viable cells was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [17].

3. Results

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

Considering that mGlu3R activation increases Aβ clearance by astrocytes [9], and SR-A plays a main role in the decline in Aβ removal during AD [11], 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 mRNA and protein levels of SR-A increased after astrocyte treatment with the mGlu3R agonist LY379268 (0.1 μM, Figure 1a-b).

Since sAPPα has been linked to enhanced SR-A -mediated Aβ uptake by microglial cells [8], we next studied the effect of recombinant sAPPα on astroglial SR-A levels. Although mRNA levels remained unchanged (Figure 1a), SR-A protein expression was significantly increased by 1 nM sAPPα (Figure 1b).

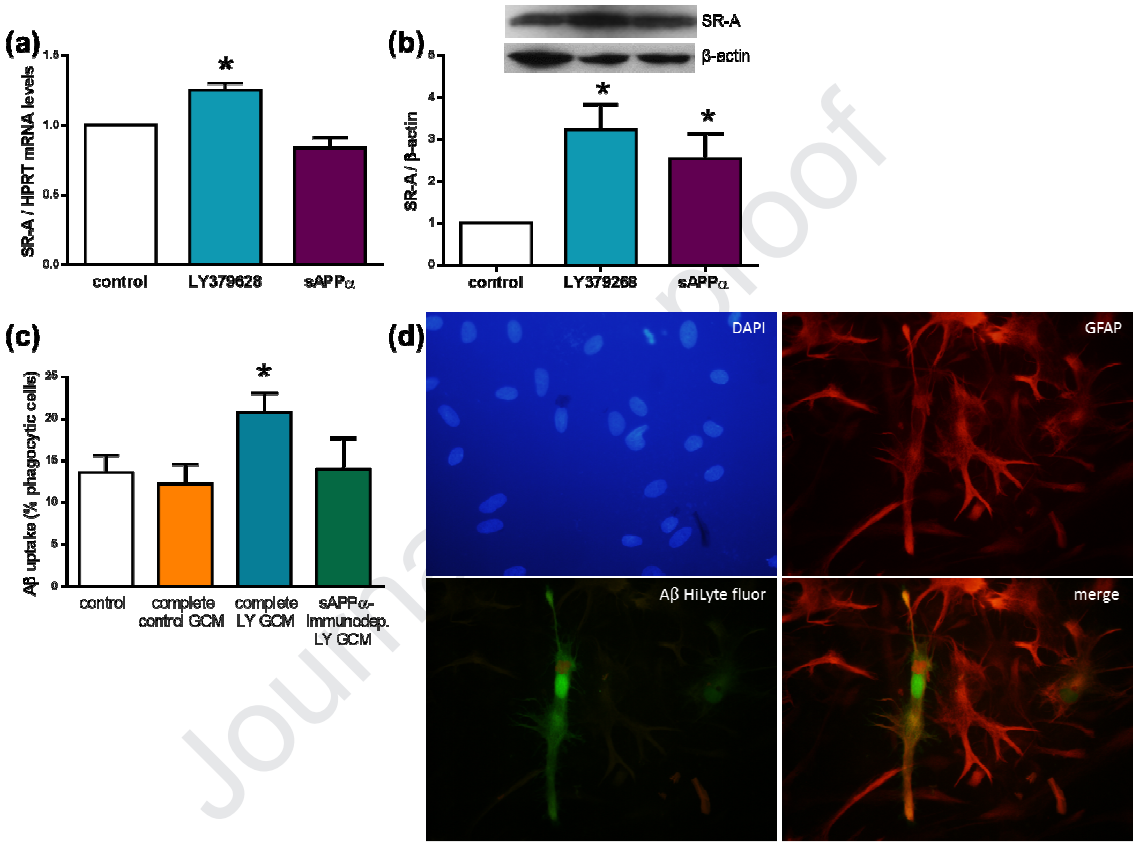

Figure 1

3.2. Release of sAPPα after mGlu3R activation mediated Aβ clearance

sAPPα involvement in mGlu3R-induced Aβ 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 $A\beta_{25-35}$ by naïve astrocytes by 60% compared to complete control-GCM (Figure 1c). However, when LY-GCM was immunodepleted of sAPPα, the aforementioned stimulatory effect was lost (Figure 1c), evidencing an autocrine or paracrine role of sAPPα in the mGlu3R-induced Aβ uptake by astrocytes. Although an effect of remnant LY379268 in the GCM on naïve astrocytes could be possible in these experiments, the fact that sAPPα depletion from LY-GCM completely inhibited the LY-GCM-induced Aβ uptake allows us to discard this possibility. Moreover, we previously reported that LY-GCM subjected to IP in absence of anti-sAPPα antibody did not modify the effect of complete LY-GCM on neuron viability [9], thereby discarding any technical artifact of immunoprecipitation in the observed biological effects.

As seen earlier for Aβ1-42 [18, 19], Aβ25-35 was localized post engulfment in both astrocyte cytoplasm and nucleus (Figure 1d).

3.3. SR-A involvement in mGlu3R- and sAPPα-induced Aβ uptake

To define the participation of SR-A in mGlu3R-induced Aβ uptake, fluorescently-labeled Aβ25-35 phagocytosis was measured in primary cultured astrocytes from wild-type and SR-A knockout mice. After treating astrocytes with LY379628 (0.1 μM) or sAPPα (1 or 10 nM), medium was replaced by fresh medium containing 5 μM Aβ25-35 HiLyte fluor488. After 24h, Aβ uptake was determined by fluorescent microscopy and expressed as the percentage of phagocytic cells in the total counted cells. Whereas all treatments increased Aβ phagocytosis in wild-type astrocytes, none affected Aβ uptake in SR-A-deficient astrocytes (Figure 2a). This result was also corroborated by measuring the Aβ remaining in culture media (data not shown).

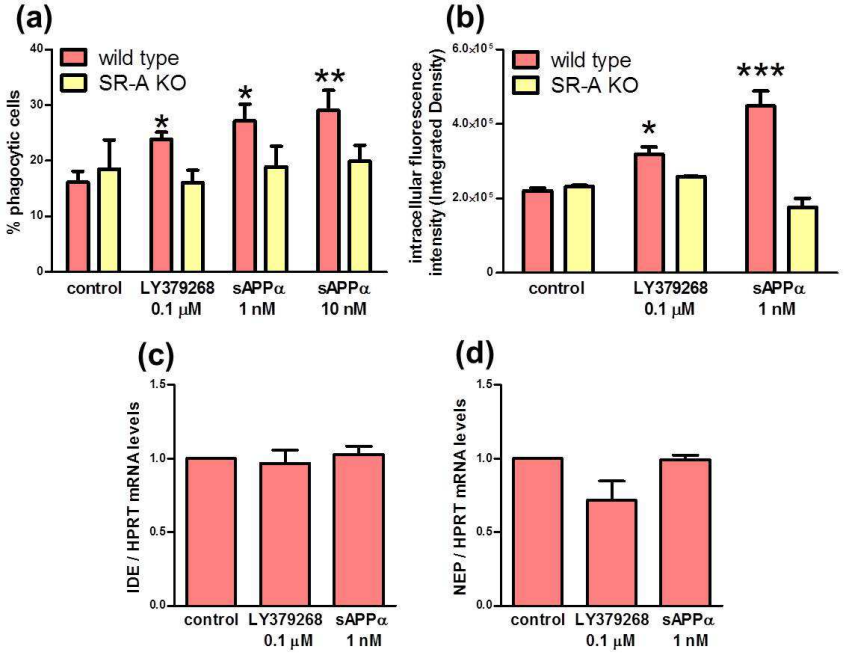

Figure 2

Quantification of intracellular fluorescence in phagocytic cells revealed increased fluorescence intensity in LY379268 and sAPPα groups for wild-type astrocytes (Figure 2b). As expected, none of the treatments affected intracellular fluorescence levels in SR-A-deficient astrocytes (Figure 2b). Therefore, LY379268- and sAPPα-induced increase of Aβ 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 Aβ degrading enzymes in wild-type astrocytes: insulin-degrading enzyme (IDE) and neprilysin (NEP). Surprisingly, neither LY379268 nor sAPPα modified mRNA levels of these catalytic enzymes (Figure 2c-d). Therefore, mGlu3R/sAPPα pathway might promote Aβ uptake and accumulation into astrocytes rather than its intracellular degradation.

3.4. The presence of residual sAPPα in supernatants inhibited Aβ uptake

An interesting and surprising finding emerged when we compared results from phagocytosis assays performed by two different approaches: first, as described in Figure 2, after treating astrocytes with

LY379628 or sAPPα, medium was completely replaced by fresh medium containing fluorescent Aβ, and Aβ uptake was determined 24h later (Figure 3a). Alternatively, after treating astrocytes with LY379628 or sAPPα, fluorescent Aβ was added to the culture medium containing incubation drugs (Figure 3b). In both cases, the control group was set as 100% and data relativized to 100%.

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

Considering that sAPPα can interact with SR-A [20] as well as with Aβ [21], one possible explanation for our observations could be that, in the second case, residual sAPPα present in the medium (both recombinant and endogenous sAPPα released in response to LY379268) was binding to Aβ and sequestering it, thereby inhibiting Aβ interaction with SR-A, a scenario schematically represented in Figure 3c.

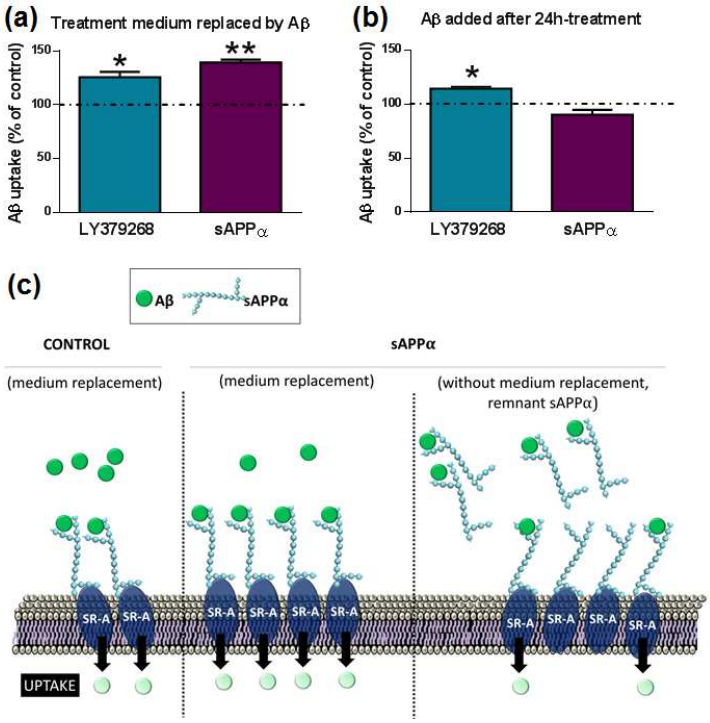

Figure 3

In order to address this issue, we designed an alternative experimental scheme (Figure 4a): astrocytes were incubated with vehicle (1st and 4th column) or sAPPα (2nd and 3rd column) for 24h; then, supernatant was replaced by fluorescent Aβ (1st and 2nd column) or by a mixture of sAPPα + fluorescent Aβ previously incubated for 1h at 37°C to allow for their interaction (3rd and 4th column), as described by Darlington et al. [21]. After a further 24h, Aβ uptake was analyzed. Our results show that Aβ/sAPPα complexes (obtained after *in vitro* heterodimerization) inhibited Aβ uptake in astrocytes pre-treated with sAPPα (Figure 4a-b, 3rd column) versus the sAPPα group (Figure 4a-b, 2nd column). However, when astrocytes were not pre-treated with sAPPα (therefore presumably not saturated SR-A) the mentioned inhibitory effect of Aβ/sAPPα complexes was reversed (Figure 4a-b, 4th column). On the contrary, Aβ/sAPPα complexes were unable to change Aβ uptake when SR-A was blocked with an anti-SR-A antibody (Figure 4a-b, 5th column). Altogether,

these results indicate that free and SR-A-associated sAPPα may compete for Aβ binding, further suggesting that sAPPα binding to SR-A may enhance SR-A dependent Aβ uptake.

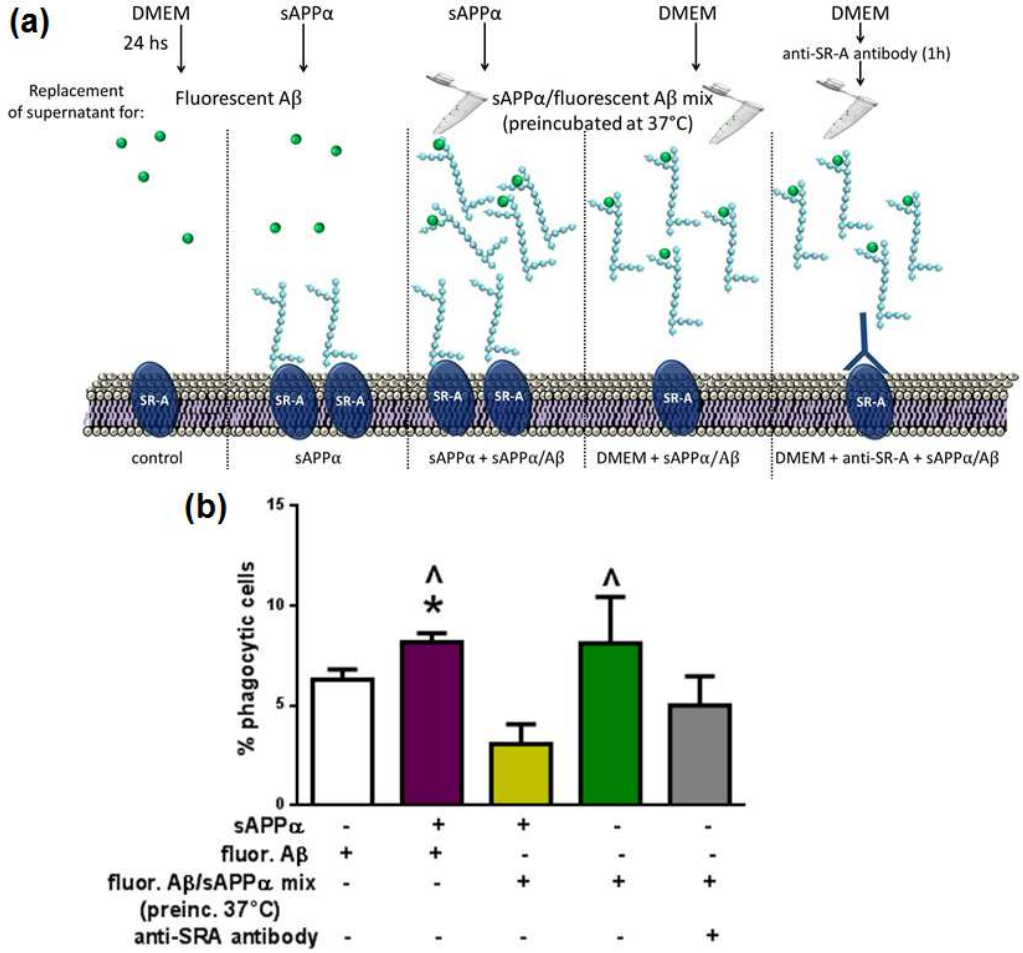

Figure 4

3.5. sAPPα/SR-A dimerization

Interaction between sAPPα 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 sAPPα (Figure 5a). We detected a 75 KDa-band corresponding to sAPPα in SR-A-precipitated samples (red arrowhead), which evidences direct interaction between these proteins. Since sAPPα is undetectable in the input sample (cellular lysates), recombinant sAPPα was run as a positive control. As an IP control, the same membrane was stripped and assayed by immunoblot for SR-A (Figure 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.

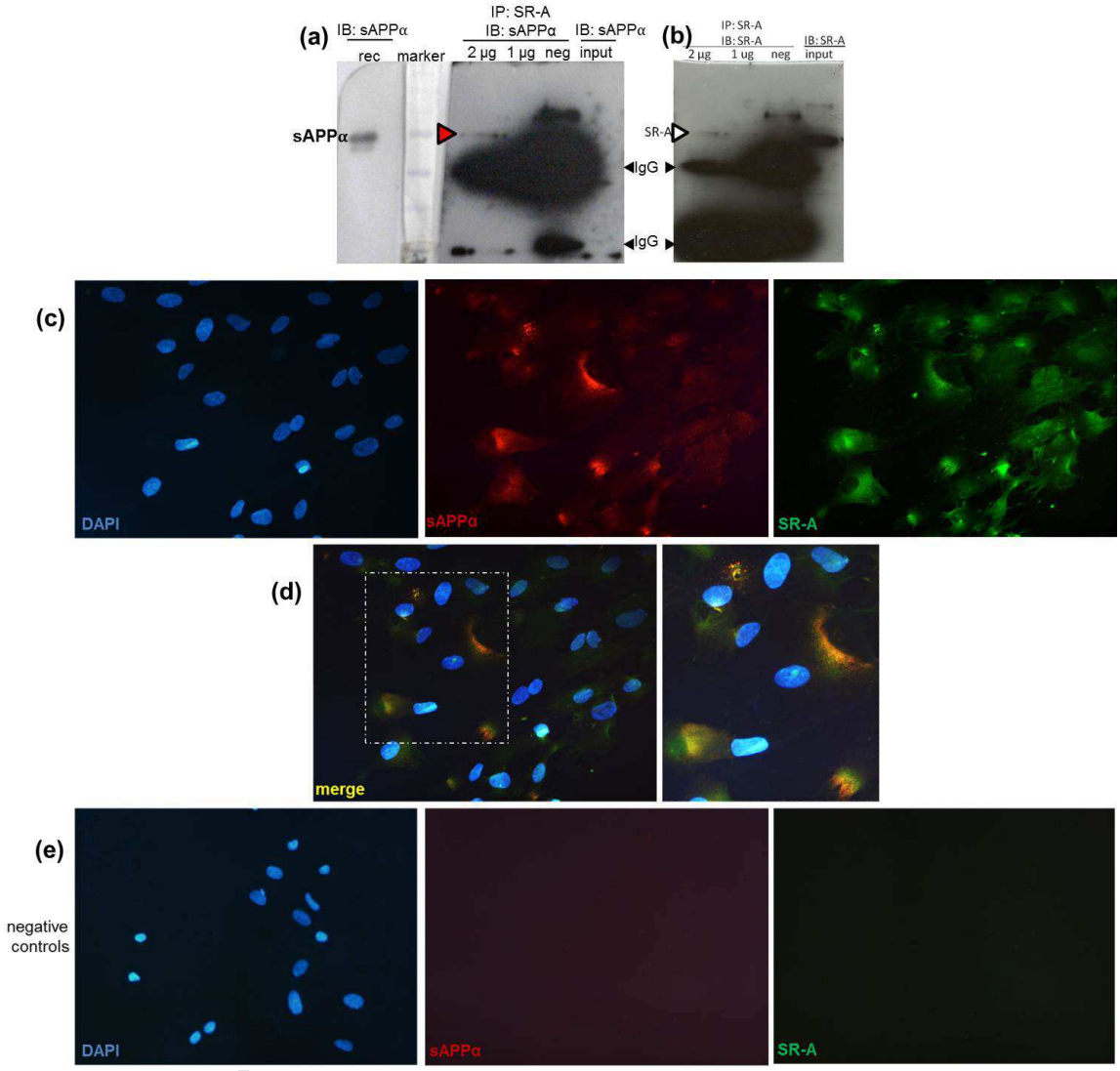

Figure 5

We also detected SR-A and sAPPα in non-permeabilized astrocytes by immunofluorescence, indicating that these proteins are associated with the plasma membrane (Figure 5c-e). Using double immunofluorescence labeling, we observed that SR-A and sAPPα were closely localized in the astrocyte plasma membrane (Figure 5c-d). A punctuate staining pattern seen for sAPPα 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 sAPPα in membrane microdomains or rafts in other cell types [22, 23].

3.6. Astrocyte viability was not affected by sAPPα

To investigate whether sAPPα can influence other cellular processes, beyond its particular autocrine/paracrine role in Aβ uptake by astrocytes, we analyzed sAPPα effect on astrocyte viability after Aβ exposure. Cell viability was significantly decreased by 25 µM $A\beta_{25-35}$ (Figure 6a-b). The detrimental effect of Aβ on cell viability was independent of its aggregation status and was not modified by either 1 or 10 nM sAPPα (Figure 6a-b). sAPPα had no effect *per se* on astroglial viability (Figure 6a-b). Therefore,

autocrine/paracrine action of sAPPα in astrocytes appears to affect Aβ elimination, but does not protect cells from Aβ toxicity, which differs from our previous results for neurons [9].

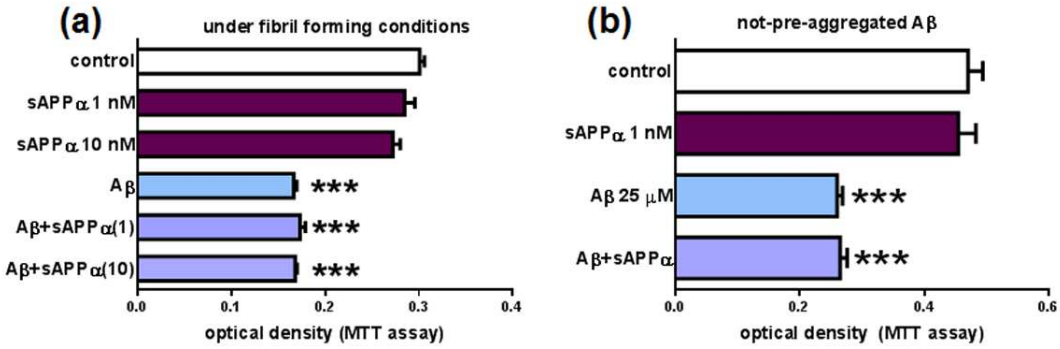

Figure 6

4. Discussion

In the field of AD, much interest is focused on Aβ toxicity, and most proposed therapies aim to mitigate Aβ production. However, it is likely that parallel deficiency in non-amyloidogenic cleavage (and consequently, ineffective sAPPα 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 [11, 24]. In the present study, we demonstrated that sAPPα produced by astrocytes in response to mGlu3R activation could act in a paracrine or autocrine fashion to stimulate Aβ uptake by astrocytes. We also showed that sAPPα- and mGlu3R-induced Aβ internalization by astrocytes depends on SR-A participation; we further suggest that sAPPα might dimerize with SR-A to enhance Aβ uptake. These results complement our previous work showing that astrocyte-derived sAPPα released after mGlu3R activation prevents hippocampal neuron death induced by Aβ [9], and contribute to consolidate the mGlu3R/sAPPα pathway as a relevant therapeutic target for AD.

Whereas Aβ production is accelerated in many familial forms of early-onset AD, increasing evidence indicates that impaired clearance of Aβ is more evident in late-onset AD [25]. Despite a long delay in recognizing astrocyte phagocytic potential compared to microglia or macrophages, the participation of plaque-associated astrocytes in Aβ elimination from AD brains is now well known. Unlike microglia, a fundamental singularity of astrocytes in the Aβ degradation process is their ability to internalize and degrade Aβ without requiring pre- or co-stimulation by cytokines [26]. Several surface mediators have been suggested as putative Aβ-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 Aβ elimination. Frenkel, et al. [12] showed that SR-A deficiency clearly impairs Aβ clearance and reduces NEP and IDE expression in the APP/PS1 AD model. Concordantly, SR-A mediates oligomeric Aβ internalization and further lysosomal degradation by cathepsin B in microglia [27]. A reduction in hippocampal SR-A expression with age has also been reported, appearing earlier in APP/PS1 mice than in wild-type animals [11]. 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 [11]. Iram, et al. [28] demonstrated that adult

astrocytes isolated from old 5xFAD mice show impaired Aβ clearance and that incubation of Aβ 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 sAPPα induced SR-A protein expression and SR-A-dependent Aβ uptake. However, these findings refer to mGlu3R- and/or sAPPα-induced Aβ uptake by postnatal 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 Aβ uptake, as suggested by Husemann, et al. [29]. However, our results do not support a redundancy mechanism scenario since SR-A depletion completely blocked mGlu3R- and sAPPα-induced Aβ phagocytosis. Our present results further show that SR-A-deficient astrocytes retained their basal phagocytic capacity although they lacked response to LY379268 or sAPPα stimuli, a finding that might tally with reported lack of SR-A involvement in basal Aβ phagocytosis. Therefore, we are hereby characterizing a pathway that might be specific for mGlu3R/sAPPα-stimulated Aβ clearance.

The therapeutic potential of sAPPα has been highlighted since the discovery of its neurotrophic and neuroprotective actions on neural cells (reviewed in [8]). Aside from its direct neurotrophic action, sAPPα can allosterically bind and inhibit β-secretase, leading to reduced Aβ synthesis [30, 31]. sAPPα also reduces tau hyperphosphorylation and GSK3β activity in neural cells and in the APP/PS1 model [32], and can improve cognition (reviewed in [8]). sAPPα enhances SR-A -mediated Aβ phagocytosis by microglia and monocytes [21], and sAPPα overexpression in APP/PS1ΔE9 mice rescued structural, electrophysiological, and behavioral deficits in these mice through microglial activation, and increased Aβ clearance [13]. However, although we know that astrocytes can also secrete sAPPα, which then enhances neuron survival [8, 9], autocrine or paracrine effects of this peptide on astroglial cells have not been exhaustively explored. We show here for the first time that sAPPα promotes Aβ clearance by cultured astrocytes. Moreover, sAPPα release by astrocytes after mGlu3R stimulation is required for mGlu3R-induced astroglial Aβ uptake, since sAPPα-depleted conditioned medium from LY-treated astrocytes did not induce Aβ uptake by naïve astrocytes, as did complete LY-GCM. This result was corroborated by using recombinant sAPPα, which was also able to improve Aβ uptake by both rat and mouse astrocytes. After this uptake, we observed an accumulation of Aβ within astrocytes, indicated by increased intracellular fluorescence in the phagocytic cells, and found no changes in mRNA levels of the two most important glial Aβ degrading enzymes, IDE and NEP. Concordant with these observations, Söllvander, et al. [33] demonstrated that astrocytes do not degrade but instead store engulfed Aβ protofibrils, since protofibrils persist for a long time in the cytoplasm of cultured astrocytes.

Better understanding of sAPPα functions and binding partners is needed in order to comprehend its neuroprotective role. Interestingly, Hoffmann, et al. [22] found that sAPP binding at the cell surface is saturable, specific, and dependent on cell differentiation, occurring in patches or microdomains. Signaling transduction after sAPPα 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 Aβ by human astrocytes may depend on Aβ conformation as well as on its association with several amyloid-associated proteins [34]. Our present results suggest that SR-A binds sAPPα and that this complex, in turn, binds Aβ to allow for Aβ clearance. Alternatively, sAPPα could associate with Aβ and then bind to SR-A to promote Aβ phagocytosis. However, sAPPα/Aβ complexes might be unable to attach to the cell when SR-A are pre-occupied by sAPPα. We further demonstrated sAPPα-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 sAPPα specifically after using clone 2B3 sAPPα

antibody, which does not bind full length APP or sAPPβ, as stated by the manufacturer. Altogether, our data support the hypothesis that sAPPα functions as a carrier that reinforces binding of Aβ to SR-A, thereby enhancing Aβ attachment and uptake by astrocytes. Previous evidence agrees with this hypothesis. Aβ is able to bind APP-derived peptides [21, 35, 36]. All SR-A ligands are polyanions, and sAPPα has a negatively charged region thought to be responsible for binding to SR-A [20]. Providing strong support for our findings, Darlington, et al. [21] have shown that, whereas aged monocytes display ineffective phagocytosis of Aβ, pretreatment of aged monocytes with sAPPα restores their phagocytic potential. They also suggested that sAPPα could form a heterodimer with Aβ, thereby promoting binding of these heterodimers to monocyte scavenger receptors and enhancing Aβ clearance. Similar to our hypothesis, results from Zhang, et al. [37] indicate that heterodimerization between $A\beta_{1-42}$ and peptide XD4 may be a prerequisite for enhanced internalization of $A\beta_{1-42}$ by microglia and, further, that $A\beta_{1-42}$ may be internalized as $A\beta_{1-42}$–XD4 complex. Moreover, XD4-induced $A\beta_{1-42}$ phagocytosis by microglia is dependent on SR-A.

Finally, we show here that $A\beta_{25-35}$ (the shortest Aβ peptide displaying neurotoxicity [38]) exerted a toxic effect on cultured astrocytes, as previously reported [19]. This effect was independent of the state of aggregation of the peptide, since both fibrillar and non fibrillar $A\beta_{25-35}$ induced similar levels of astrocyte death. In both cases, recombinant sAPPα had no effect on astrocyte viability, a finding that contrasts with the reported pro-survival effect of sAPPα on hippocampal neurons [9].

5. Conclusion

Growing knowledge of astroglial involvement in Aβ plaque clearance encourages research oriented towards restoring Aβ 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 Aβ from AD brains. Our present results show that the mGlu3R/sAPPα pathway stimulates Aβ clearance by astrocytes in an SR-A-dependent fashion. We also suggest a double binding of sAPPα to SR-A and Aβ as the mechanism underlying Aβ clearance enhancement by mGlu3R and sAPPα. 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 sAPPα offers great therapeutic potential, it is too large to cross the BBB [8]. Another predictable way to increase sAPPα 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 sAPPα from astrocytes.

6. 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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9. Legends

Figure 1. sAPPα involvement in Aβ clearance by astrocytes. Rat primary astrocyte cultures were incubated with the mGlu3R agonist LY379268 (0.1 μM) or recombinant sAPPα (1 nM) for 24h 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 ± 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 3h, then glial conditioned medium (GCM) was collected and half volume subjected to sAPPα immunodepletion using an anti-sAPPα antibody and protein A/G agarose. Naïve astrocytes were incubated for 24h with complete control GCM, complete LY GCM, or sAPPα-immunodepleted LY GCM, followed by a further 24h treatment with 5 μM $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$. Aβ 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 ± 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).

Figure 2. mGlu3R- and sAPPα-induced Aβ 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 μM LY379268 or 1-10 nM recombinant sAPPα for 24h. Then medium was replaced by fresh medium containing 5 μM $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ for a further 24h. Aβ 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 ± SE from 3-4 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

μM LY379268 or 1 nM recombinant sAPPα for 24h and mRNA levels of IDE (c) and NEP (d) were determined by real-time RT-PCR. Data were expressed as RQ mean ± SE from 2 independent experiments.

Figure 3. Residual sAPPα in supernatants inhibits Aβ uptake by astrocytes. Astrocytes were treated with vehicle, LY379268, or sAPPα for 24h and then either the medium was replaced by fresh medium containing 5 μM $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ (a) or else the $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ was added directly to the culture media containing the drugs (b). After 24h, Aβ 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 sAPPα and this, in turn, binds to Aβ, then the presence of free sAPPα in the culture media would compete with SR-A-bound sAPPα for Aβ binding, thereby avoiding Aβ clearance.

Figure 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 24h. Then, medium was replaced by $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ for a further 24h; first column in (b)
'sAPPα' group: astrocytes were treated with sAPPα for 24h. Then, medium was replaced by $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ for a further 24h; second column in (b)
'sAPPα + sAPPα/Aβ' group: astrocytes were treated with sAPPα for 24h. Then, medium was replaced by fresh medium containing $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ and sAPPα (previously incubated at 37°C for 1h) for a further 24h; third column in (b)
'DMEM + sAPPα/Aβ' group: astrocytes were treated with vehicle for 24h. Then, medium was replaced by fresh medium containing $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ and sAPPα (previously incubated at 37°C for 1h) for a further 24h; fourth column in (b)
'DMEM + anti-SR-A + sAPPα/Aβ' group: astrocytes were treated with vehicle for 24h. Then, medium was replaced by anti-SR-A antibody (0.8 μg/ml) to block SR-A for 1h, then medium was replaced by fresh medium containing $A\beta_{25\text{-}35}$ HiLyte $fluor^{488}$ and sAPPα (previously incubated at 37°C for 1h) for a further 24h; 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)

Figure 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.

Figure 6. The detrimental effect of Aβ on astrocyte viability is not prevented by sAPPα. Astrocytes were incubated with 25 μM $A\beta_{25-35}$ either as fibrils (pre-aggregated Aβ at 37°C for 3 days) (a) or without pre-aggregation (b) in the presence or absence of sAPPα (1-10 nM) for 24h. 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).

Highlights

- sAPPα released by astrocytes after mGlu3R stimulation induces Aβ uptake
- mGlu3R and sAPPα-induced Aβ clearance by astrocytes depend on SR-A participation
- Astrocytes appear to accumulate rather than degrade phagocyted Aβ
- sAPPα can dimerize with SR-A at the astroglial surface
- sAPPα might serve as a carrier for Aβ to enhance SR-A-mediated Aβ clearance