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Astroglial mGlu3 receptors promote alpha-secretase-mediated amyloid precursor protein cleavage

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ABSTRACT

Amyloid precursor protein (APP) shedding yields the Alzheimer's disease (AD)-related peptide amyloid β (Aβ) through β- and γ-secretase cleavage. Alternatively, α-secretase cleavage generates a soluble and neuroprotective fragment (sAPPα) while precludes the production of Aβ. Although metabotropic glutamate (mGlu) receptors were associated with induction of sAPPα production in astrocytes, there was no further evidence regarding the specific subtype receptor or the mechanisms involved in this action. In the present study, we used the dual mGlu2/3 receptor agonist LY379268, which in pure astrocyte cultures selectively activates mGlu3 receptor subtype since mGlu2 receptor subtype is not expressed by these cells. We showed that LY379268 incremented sAPPα release from cultured astrocytes by inducing α-secretases expression, whereas it decreased β-secretase levels. LY379268-induced increase of PPAR-γ levels could be involved in the effect of the agonist on sAPPα release. Using the PDAPP-J20 murine model of AD we described a strong reduction in mGlu2/3 receptor expression in the hippocampus of 5- and 14-month-old transgenic mice compared to control littermates. Moreover, mGlu3 receptor expression is also decreased specifically in hippocampal astrocytes of these transgenic animals as a function of age. Therefore, diminished levels of hippocampal mGlu3 receptors might have implications in the development of the disease in these transgenic mice considering the anti-amyloidogenic action of mGlu3 receptors in astrocytes.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative process affecting a growing number of aged people as life expectancy increases. Its early, hereditary form accounts for only 1–6% of the cases, whereas the sporadic, most frequent form appears generally after 65 years of age and is enhanced by risk factors such as hypertension, high plasmatic cholesterol, diabetes, head injury or low educational levels (Patterson et al., 2008). AD is histologically characterized by extracellular deposits of amyloid β (Aβ) also called senile plaques, intracellular accumulation of tangles of phosphorylated tau protein, gliosis, oxidative damage, neuron death and synapse loss, thereby resulting in cognitive deficits (Chong et al., 2005; Heneka et al., 2005). Amyloid β accumulation might result from decreased elimination from the brain as well as from increased production through sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. Alternatively, in non-pathological conditions, non-amyloidogenic cleavage of APP by α- and γ-secretases generates the carboxyl-truncated soluble product of APP cleavage by α-secretase (sAPPα) and the non-amyloidogenic 3 kDa peptide (p3) instead of intact 4 kDa Aβ (Thinakaran and Koo, 2008). Indeed, sAPPα has been reported to show neurotrophic and neuroprotective actions (Bailey et al., 2011; Corrigan et al., 2011; Gakhar-Koppole et al., 2008; Mattson, 1997; Stein and Johnson, 2003; Turner et al., 2003) and to improve memory performance in both normal and amnesic mice (Meziane et al., 1998). Moreover, sAPPα is able to bind and inhibit the β-secretase BACE1 and to revert amyloidosis in APP transgenic mice (Obregon et al., 2012). Some proteins belonging to the A Disintegrin And Metalloprotease (ADAM) family were found to show α-

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secretase activity; of these, ADAM10 and ADAM17 are the most abundant in the brain and were identified as the main APP proteases *in vivo* (Jorissen et al., 2010; Postina et al., 2004; Weskamp et al., 2002). In APP transgenic mice, ADAM10 overexpression induces sAPPα secretion, reduces Aβ production, and ameliorates cognitive deficit (Postina et al., 2004).

Astrocyte participation in AD development has not yet been fully elucidated. Although reactive gliosis can enhance Aβ toxicity by increasing cytokine production and oxidative damage (Akiyama et al., 2000; Luth et al., 2001; Shibata and Kobayashi, 2008; Wyss-Coray et al., 2003), it is also thought that astroglial atrophy occurring at early stages of the disease could precede plaques and tangles formation (Rodriguez et al., 2009). Recently, we reported significant astroglial morphological changes in the hippocampus of PDAPP-J20 mice (Beauquis et al., 2013). Whereas plaque-associated GFAP$^+$ astrocytes exhibited greatly increased volume and an inflammatory phenotype, non-plaque-associated astrocytes showed lower volume and increased ramifications compared to non-transgenic hippocampal astrocytes (Beauquis et al., 2013). Remarkably, in the hippocampal stratum radiatum from 5-month-old transgenic mice, when no amyloid deposits were present, we found fewer astroglial cells than in control mice, suggesting an early onset of glial alterations in AD pathogenesis (Beauquis et al., 2013). Thus, it is possible that astrocytes initially have a protective role by modulating not only Aβ clearance (Funato et al., 1998; Leal et al., 2006; Shaffer et al., 1995; Thal et al., 1999) but also AD-related glutamate excitotoxicity (Bruno et al., 1998; Wilson, 1997; Yao et al., 2005), whereas as AD progresses neurotoxicity caused by excessive gliosis seems to prevail over neuroprotection.

Metabotropic glutamate (mGlu) receptors are classified into three groups: group I (mGlu1/5), associated with phosphatidyl inositol hydrolysis; and groups II (mGlu2/3) and III (mGlu4/6/7/8) which are negatively coupled to adenylate cyclase and to cyclic AMP (cAMP) formation. mGlu2/3 receptor agonists show protective actions against excitotoxic and apoptotic stimuli *in vitro* (Buisson and Choi, 1995; Ciccarelli et al., 2007; Copani et al., 1995; Durand et al., 2010, 2011; Kingston et al., 1999; Matarredona et al., 2001) and *in vivo* (Bond et al., 2000; Miyamoto et al., 1997). Some authors have also linked these receptors to AD. Mutations in mGlu3 receptor gene (GRM3) affect hippocampal function and memory in humans (de Quervain and Papassotiropoulos, 2006; Egan et al., 2004). In HEK293 cells GRM3 trans-expression induces sAPPα secretion (Schobel et al., 2006), whereas in astrocyte cultures a non-selective agonist of group I/II mGlu receptors (1-aminocyclopentane-1,3-dicarboxylic acid, ACPD) promotes the non-amyloidogenic cleavage of APP (Lee and Wurtman, 1997). The latter effect was blocked by cAMP (Lee and Wurtman, 1997), suggesting that adenylate cyclase-negatively-coupled mGlu receptors may be responsible for this anti-amyloidogenic action, although no further studies using newer selective mGlu2/3 receptor agonists have been published. Recently, Caraci et al. (2011a) demonstrated that the mGlu2/3 receptor selective agonist (1*R*,4*R*,5*S*,6*R*)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) reduced Aβ-induced neurodegeneration in mixed neuro-glial cultures, an effect abolished by an mGlu3 receptor negative allosteric modulator, whereas LY379268 lost its neuroprotective activity either in pure neuronal cultures or in mixed cultures containing astrocytes from mGlu3R$^{-/-}$ mice. These data indicate that activation of glial mGlu3 receptors results in neuroprotection against Aβ.

Considering all this evidence, the aim of the present study was to investigate the effect of selective mGlu3 receptor activation on APP proteolytic cleavage in cultured rat astrocytes and to explore possible mechanisms involved in this effect. On the other hand, data about group II mGlu receptor expression in AD brains or animal models are not only limited but also far from enlightening, since they are highly dependent on the brain region analyzed, the model, and the analytical method applied (Cha et al., 2001; Dewar et al., 1991; Lee et al., 2004; Richards et al., 2010). Therefore, we were also interested in studying mGlu3 receptor expression in the hippocampus of PDAPP-J20 mice, a well-established AD model.

2. Materials and methods

2.1. Reagents

LY379268 and 2-Chloro-5-nitro-*N*-phenylbenzamide (GW9662) were purchased from Tocris Bioscience (MO, USA). Unspecified reagent source was Sigma–Aldrich Corporation (MO, USA).

2.2. Animals

Transgenic PDAPP-J20 mice carrying the human APP gene with Swedish and Indiana mutations (Hsia et al., 1999; Mucke et al., 2000) were maintained by heterozygous crosses with C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) in the animal facility of the Institute of Biology and Experimental Medicine (IBYME, UBA-CONICET; NIH Assurance Certificate #A5072-01). Transgenicity was confirmed by RT-PCR using primers for hAPP. Female PDAPP-J20 mice (Tg) and their non-transgenic littermates (NTg) were housed in groups of 4 under controlled conditions of temperature (22 °C) and humidity (50%) with 12 h/12 h light/dark cycles and were euthanized at 5 or 14 months of age using a combination of ketamine (80 mg/kg BW, i.p.; Holliday-Scott, Argentina) and xylazine (10 mg/kg BW, i.p.; Bayer, Argentina) followed by transcardial perfusion with 30 mL of 0.9% saline and 30 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After overnight fixation with 4% paraformaldehyde, brains were cut coronally at 60 μm using a vibrating microtome and cryopreserved at −20 °C until use. Procedures in animals were done following the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Institute of Biology and Experimental Medicine. All efforts were made to minimize animal suffering and number of mice used.

2.3. Cell cultures

Astrocytes were prepared from rat cerebral hemispheres of 1- to 2-day-old postnatal Wistar rat pups. Hemispheres were dissected, freed from meninges, and cut into small fragments. The tissue was disrupted by triturating it through a needle and cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen Life Technologies, CA, USA) containing 10% fetal bovine serum (FBS, Natocor, Argentina), 50 μg/mL streptomycin and 50 U penicillin (Invitrogen Life Technologies, CA, USA) in 75 cm^2 poly-L-lysine coated culture flasks at 37 °C in 5% CO_2. Once grown to confluence, astrocytes were separated from microglia and oligodendrocytes by shaking overnight at 200 rpm. Cells were trypsinized, subcultured, and after 2–3 days of stabilization, incubated with the drugs in minimal essential medium (MEM) (Sigma–Aldrich Corporation, MO, USA) containing 6 mM L-glutamine, 50 μg/mL streptomycin and 50 U penicillin without FBS. Cultures were routinely more than 95% pure astrocytes as assessed by glial fibrillary acidic protein (GFAP) immunostaining. Experimental procedures were carried out with approval by the Committee on Ethics of the University of Buenos Aires Medical School.

2.4. sAPPα ELISA

sAPPα levels in astrocyte culture supernatants were quantified with a solid phase sandwich ELISA kit (Immuno-Biological Laboratories America, MN, USA) following the manufacturer's instructions. Cultured astrocytes (250 cells/μL) were incubated with LY379268 (0.01–10 μM) or with 0.1 μM LY379268 ± 2 μM GW9662 in serum-free MEM for 3 h. When treated with GW9662, astrocytes were preincubated with GW9662 for 15 min followed by incubation with LY379268 + GW9662. One hundred μL of culture supernatant were used for ELISA assays. Optical density (OD) was measured in a microplate spectrophotometer (BioRad Laboratories, CA, USA) at 450 nm. sAPPα concentration was determined from a sAPPα standard curve and expressed as pg/mL.

2.5. Reverse transcription – real time polymerase chain reaction (RTqPCR)

Cultured astrocytes (1×10^6 cells) were treated with LY379268 (0.1–10 μM) for 3 or 16 h in serum-free MEM. Total RNA was extracted using TRIZOL reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's protocol. 2 μg of total RNA were treated with 1 U RQ1 RNAse free-DNAse (Promega Corporation, WI, USA) at 37 °C for 10 min. RT reaction was carried out using 0.4 μg oligo-dT primers (Invitrogen Life Technologies, CA, USA) plus 1 μL ImProm-II™ Reverse Transcriptase (Promega) for 1 h at 42 °C with 3 mM MgCl. The rat ADAM10 (accession number Z48444), ADAM17 (NM-020306.1) and beta-site APP cleaving enzyme 1 (BACE1, AF-190727) genes were analyzed using the following PCR primers set: ADAM10 forward 5′-TCCCAAGCCCAACTTTACAGA-3′, ADAM10 reverse 5′-TGCACATTGCCCATTAATGC-3′, ADAM17 forward 5′-TGGAGTCCTGCGCATGTG-3′, ADAM17 reverse 5′-CATGGGC-CAGAAAGGTTCCT-3′, BACE1 forward 5′-TTGCCATGTGCACGATGAG-3′, BACE1 reverse

5′- GCCGTGACAAACGGACCTT-3′. HPRT was used as endogenous control (HPRT forward 5′-CTCATGGACTGATTATGGACAGGAC-3′, HPRT reverse 5′-CAGGTCAGCAAAGAACTTATAGCC-3′). Real-time PCR amplifications were done in a StepOne™ Real-Time PCR System (Applied Biosystem) and PCR reactions were set up in a final volume of 16 μL containing 4 μL of cDNA (1:20 dilution), 900 nM (for ADAM10) or 450 nM of primers (for ADAM17, BACE1 and HPRT) and SYBR Green Master Mix (Applied Biosystem – Life Technologies Co, CA, USA). PCR conditions were denaturation at 95 °C for 10 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 ADAM and BACE expression were normalized to the endogenous control gene HPRT and analyzed with Step-One Software (Applied Biosystems) using the comparative ΔΔCt method (Livak and Schmittgen, 2001). Data were reported as RQ mean of 3–4 biological replicates (with 3 technical replicates each) ± SE.

2.6. Western blot

One million astrocytes per well treated with LY379268 (0.1–10 μM) for 3 or 24 h were scrapped into PBS + 10 mM NaF + 1 mM Na_3VO_4 and centrifuged at 2000 rpm. Pellets were homogenized in lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40) containing protease and phosphatase inhibitors (1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 10 mM NaF, 1 mM Na_3VO_4) 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. Twenty five to fifty μg of protein were size-fractionated in 8–10% sodium dodecyl sulfate (SDS)-polyacrilamide gel. For ADAM determination, proteins were diluted in sample buffer containing leupeptin instead of β-mercaptoethanol as previously suggested (Tousseyn et al., 2009). Proteins were electrotransferred to a polyvinylidene difluoride membrane and blots were blocked for 2 h in 5% nonfat dry milk-TBS-0.1% Tween 20 and incubated overnight at 4 °C with appropriate primary antibodies in 5% milk-TBS-0.1% Tween 20: anti-ADAM10 H-300 (Santa Cruz Biotechnology, CA, USA) 1:200, anti-ADAM17 H-300 (Santa Cruz Biotechnology, CA, USA) 1:200, anti-PPAR-γ (Santa Cruz Biotechnology, CA, USA) 1:500, anti-GAPDH (Santa Cruz Biotechnology, CA, USA) 1:10,000. Anti-BACE antibody (Cell Signaling Technology, MA, USA) was incubated in 5% BSA-TBS-0.1% Tween 20. After 1 h incubation with the respective biotinylated secondary antibodies (Millipore Co.) and 1 h incubation with Streptavidin-peroxidase (Millipore Co.), immunoreactivity was detected by enhanced chemiluminescence (Bio-Lumina, Productos Bio-Lógicos, Argentina). ADAM10, ADAM17 and GAPDH expression was analyzed on the same membrane after stripping. Bands were analyzed using SCION Image software. Results were normalized to the internal control GAPDH and expressed as arbitrary units (AU) relative to respective controls.

2.7. Immunohistochemistry

Immunohistochemistry was done to study the expression of mGluR2/3 in the hippocampus of PDAPP-J20 mice and to determine its presence in astrocytes. Mice were grouped into four groups of four mice: 5-month-old NTg mice, 14-month-old NTg mice, 5-month-old Tg mice and 14-month-old Tg mice. The technique was performed on free-floating coronal brain sections. Every eighth coronal brain section throughout the entire rostrocaudal extension of the hippocampus was analyzed in each mouse (6 sections per brain, 4 animals per group). After rinsing to remove cryoprotectant solution, sections were exposed to methanol 50% in PBS for 10 min at room temperature. Blocking of unspecific binding sites was done by incubating with 5% normal goat serum and 0.5% triton X100 in PBS at 37 °C for 30 min. Sections were incubated overnight with the following primary antibodies: rabbit polyclonal anti-mGluR2/3 (1:500; #06-676 Millipore Co.) and mouse monoclonal anti-GFAP (1:500; MAB360 Millipore Co.). After incubation with secondary fluorescent antibodies (anti-rabbit Alexa 488 and anti-mouse Alexa 555, Invitrogen), sections were placed on gelatin-coated slides and mounted with PVA-DABCO (Sigma–Aldrich). Immunolabeled sections were imaged with a Zeiss Axioplan fluorescence microscope with a 10× objective. The region with the highest staining was the stratum lacunosum moleculare (LMol) of CA1. Regions of interest were outlined on microphotographs of the hippocampus using Optimas 6.5 software (Media Cybernetics) and OD was calculated. Also, OD of the background was analyzed in order to calculate a differential score by the formula [specific LMol OD/background OD - 1] which expresses the proportional increase of the specific OD compared to OD of the background. Also, sections were imaged with a Nikon E80 confocal microscope in order to determine the localization of mGluR3 in GFAP-positive astrocytes. Using JACoP plugin for ImageJ (Bolte and Cordelieres, 2006) we determined the co-localization of GFAP and mGlu2/3 immunofluorescence in LMol of CA1. Co-localization between both markers was calculated using Mander's coefficient (Manders et al., 1992) where 0 indicates non-overlapping and 1 corresponds to 100% co-localization between channels. The coefficient indicates the proportion of the GFAP signal coincident with the mGlu2/3 signal over GFAP total intensity. Results were expressed as the ratio of the summed intensities of pixels from the red channel (GFAP) for which the intensity in the green channel (mGlu2/3) was above zero to the total intensity in the red channel. Threshold for each channel was set to minimize interference of background noise signal.

2.8. Statistical analysis

Data were expressed as mean ± SEM and analyzed by one-way or two-way analysis of variance (ANOVA) followed by the Dunnet or Bonferroni post-test or by one- or two-sample Student's t test, when appropriate. Differences with a $p < 0.05$ were considered statistically significant.

3. Results

3.1. Astroglial mGlu3 receptor activation by LY379268 promoted sAPPα release and induced synthesis of α-secretases ADAM10 and ADAM17

In order to determine the participation of mGlu3 receptor in non-amyloidogenic shedding of APP in astrocytes, primary cultured rat pup astrocytes were incubated with group II mGluR-selective agonist LY379268 for 3 h and production of sAPPα was measured in the culture media by ELISA. All experiments were performed in serum-free medium to avoid any cross-reactivity with sAPPα determinations as per manufacturer's instructions. LY379268 increased sAPPα release by nearly 40% at all concentrations tested, although the effect of LY379268 at 1 μM did not reach statistical significance (Fig. 1). It is thought that LY379268's actions in astrocytes are mediated by mGlu3 receptor subtype, because mGlu2 subtype is not expressed by this cell type (reviewed in Durand et al., 2013) and was elegantly demonstrated by Caraci et al. (2011a) using $mGlu3R^{-/-}$ mice-derived astrocyte cultures.

Since ADAM family proteins have been found to have α-secretase activity and ADAM10 and ADAM17 are the members most present in the brain (Jorissen et al., 2010; Postina et al., 2004; Weskamp et al., 2002), we evaluated mRNA and protein levels of both proteases in the presence of LY379268. At both 3 and 16 h of incubation, LY379268 increased ADAM10 and ADAM17 mRNA levels, this effect being higher at 16 h compared to 3 h (Fig. 2a). Likewise, ADAM10 and ADAM17 protein levels were also strongly induced by this agonist (Fig. 2b).

3.2. LY379268 diminished β-secretase levels in astrocytes

Whereas non-selective mGluR activation had been associated with induction of the non-amyloidogenic pathway (Lee and Wurtman, 1997), direct action of these receptors in β-secretase has not yet been explored. Therefore, we evaluated the effect of LY379268 on β-secretase BACE1 mRNA and protein levels. Interestingly, LY379268 induced BACE1 gene expression at 3 and 16 h (Fig. 3a) but strongly reduced BACE protein levels at 24 h (Fig. 3b), suggesting the action of a regulatory mechanism at the translational level.

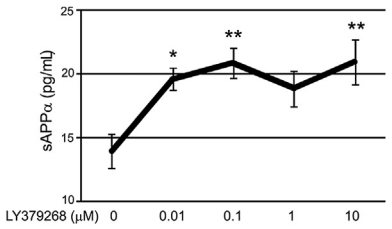

Fig. 1. Astroglial mGlu3 receptor activation induced the release of sAPPα to the culture medium. Cultured astrocytes from 1-2-day-old Wistar rat pups were incubated with the group II mGlu receptors agonist LY379268 (0.01–10 μM) for 3 h in supplemented MEM without serum and sAPPα levels in the culture medium were determined by solid phase sandwich ELISA and expressed as pg/mL. Each point represents the mean ± SEM of 4 technical replicates from 1 experiment representative of 2 independent ones. $^{*}p < 0.05$; $^{**}p < 0.01$ versus control group.

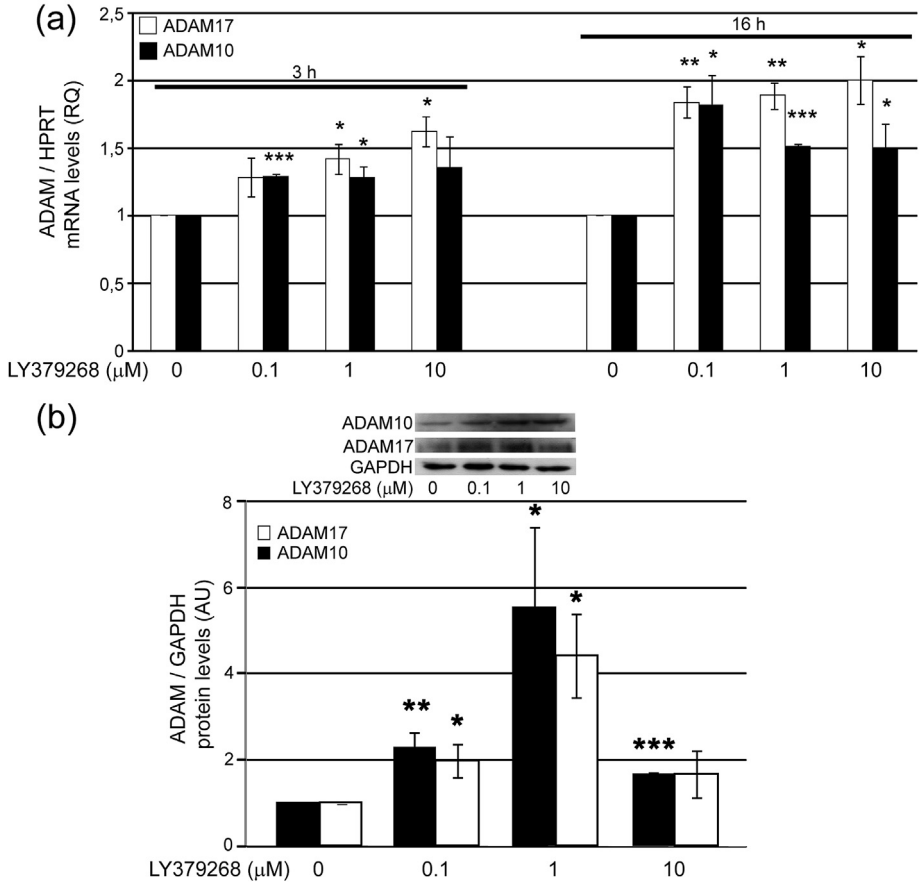

Fig. 2. LY379268 promoted ADAM10 and ADAM17 gene and protein expression. Cultured astrocytes were incubated with LY379268 (0.1–10 μM) in serum-free MEM for 3 and 16 h (a) or 24 h (b). (a) Total mRNA was extracted using TRIZOL, and mRNA levels for ADAM10 (black bars) and ADAM17 (white bars) were assayed by reverse transcription-real time-PCR (RTqPCR) in a StepOne™ Real-Time PCR System. HPRT mRNA levels were used as endogenous control. Data were analyzed by the comparative ΔΔCt method and the mean ± SEM of the RQ values from 3 to 4 biological replicates were expressed. (b) Total protein homogenates were assayed by Western blot for ADAM10 (black bars) and ADAM17 (white bars). GAPDH was used as endogenous control. All three proteins were determined on the same membrane after stripping. OD of revealed bands was measured and values normalized to GAPDH and expressed as arbitrary units (AU) relative to control group ($n = 4–5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control group.

3.3. *Involvement of peroxisome proliferator-activated receptor γ (PPAR-γ) in mGlu3 receptor effects on the non-amyloidogenic pathway*

It has been reported that PPAR-γ is involved in promoting sAPPα production and repressing BACE transcription (Sastre et al., 2008). Therefore, we analyzed whether PPAR-γ could mediate mGlu3 receptor-induced sAPPα production in astrocytes. Protein levels of PPAR-γ were significantly increased by LY379268 after 3 h of incubation, but PPAR-γ levels diminished after prolonged (24 h) exposure to the agonist (Fig. 4a), an effect that is likely related to the short half-life of PPAR-γ upon activation.

Furthermore, we found that the statistically significant effect of LY379268 on sAPPα release was not observed in the presence of the PPAR-γ antagonist GW9662 (Fig. 4b), showing a trend to reduce LY379268-induced sAPPα production. Moreover, GW9662 as well as another PPAR-γ antagonist (BADGE) were able to inhibit stimulatory action of LY379268 on both ADAM10 (Fig. 4c) and ADAM17 (Fig. 4d) protein expression. Thus, PPAR-γ might mediate effects of LY379268 on the non-amyloidogenic cleavage of APP.

3.4. *mGlu3 receptor expression in PDAPP-J20 transgenic mice*

To date, very few experiments have explored changes in group II mGlu receptor expression in Alzheimer's lesions and their results are ambiguous, whereas none of them has focused on astroglial mGlu3 receptor expression. The PDAPP-J20 Alzheimer's animal model derived from the generation of platelet-derived growth factor (PDGF) β-chain promoter-driven human APP minigene (hAPP) carrying the Swedish and Indiana familial AD mutations, with mice having aspects of AD pathology such as Aβ accumulation, neuritic plaque formation, oxidative stress, and memory deficits from 6 to 7 months of age (Robinson et al., 2011). Using this model we studied mGlu3 receptor expression in the hippocampus from 5- or 14-month-old NTg or Tg PDAPP-J20 female mice by immunohistochemistry (IHC). Using an anti-mGlu2/3 receptor antibody we were able to detect receptor expression mainly in the CA1 stratum lacunosum moleculare (LMol) (Fig. 5a), as also described by others (Pacheco Otalora et al., 2006; Shigemoto et al., 1997). Interestingly, we observed a significant reduction in the hippocampal expression of these receptors in both 5- and 14-month-old Tg mice compared to NTg controls, but also in 14-month-old NTg mice compared to 5-month-old NTg mice (Fig. 5b), evidencing a clear genotype component as well as an age-related component in the differences observed.

We later performed a double IHC for mGlu2/3 receptor and GFAP in the hippocampal slices (Fig. 5c). Considering that astrocytes express only mGlu3 receptor subtype, mGlu2/3 receptor immunostaining co-localizing with GFAP should be considered selective mGlu3 receptor staining. As shown in Fig. 5c and d, we

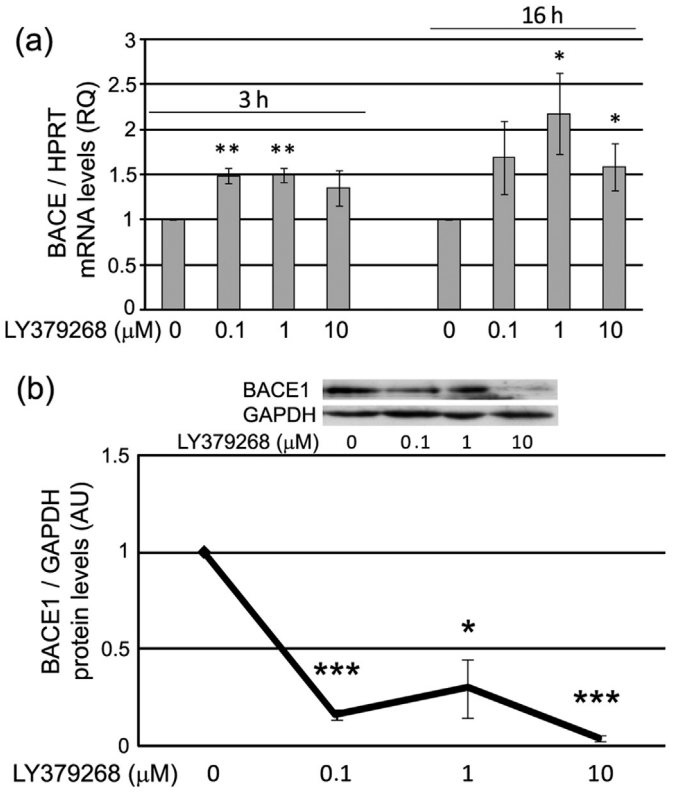

Fig. 3. LY379268 increased BACE1 mRNA but repressed BACE1 protein expression. Cultured astrocytes were incubated with LY379268 (0.1–10 μM) in serum-free MEM for 3 and 16 h (a) or 24 h (b). (a) Total mRNA was extracted using TRIZOL, and mRNA levels for BACE1 were assayed by RTqPCR. HPRT mRNA levels were used as endogenous control. Data were analyzed by the comparative ΔΔCt method and the mean ± SEM of the RQ values from 3 biological replicates were expressed. (b) Total protein homogenates were assayed by Western blot for BACE1 using GAPDH as endogenous control. OD of revealed bands was measured and values normalized to GAPDH and expressed as arbitrary units (AU) relative to control group ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control group.

found a loss of co-localization between mGlu3^{+} and GFAP^{+} staining in 14-month-old Tg mice compared to 5-month-old Tg mice, a difference which was not evidenced in total mGlu2/3 staining (Fig. 5b). Although we also observed a consistent reduction (≈24%) in mGlu3/GFAP co-localization in 14-month-old Tg mice versus age-matched NTg controls (Fig. 5d), this difference did not reach statistical significance, as did in the whole CA1 LMol (Fig. 5b). A minor reduction in astroglial mGlu3 receptor in 14- versus 5-month-old NTg mice was also observed (Fig. 5d). However, in GFAP^{+} cells it was not found reduced mGlu3 receptor expression in 5-month-old Tg mice versus age-matched NTg controls (Fig. 5d), as was observed in the whole CA1 LMol (Fig. 5b). This difference was not related to variations in astrocyte density, since the number of astroglial cells in LMol from 5-month-old Tg and NTg mice was similar (data not shown). Thus, changes specifically in astrocytic mGlu3 receptor expression might contribute to alterations in total receptor levels in CA1 LMol. However some differences observed between experimental groups are selective for astroglial or neuronal receptors.

4. Discussion

In the present report we describe a novel neuroprotective function of mGlu3 receptors related to their ability to promote the non-amyloidogenic pathway of APP cleavage in astrocytes, thus enhancing sAPPα production. Combining an *in vitro* approach with specific mGlu3 receptor immunohistochemical detection in brain slices from PDAPP-J20 mice, our results clearly showed that: (1) astroglial mGlu3 receptor activation by LY379268 induces sAPPα release and increases α-secretases ADAM10 and ADAM17 mRNA and protein levels, whereas it decreases β-secretase BACE protein levels; (2) LY379268 also induces early PPAR-γ expression; and (3) there is a loss of astroglial mGlu3 receptor expression in 14-month-old PDAPP transgenic mice. Considering the anti-amyloidogenic function of mGlu3 receptors in astrocytes *in vitro*, their altered hippocampal levels might be linked to the appearance of AD-like symptoms and senile plaques in the transgenic mice.

Several years ago, Lee and Wurtman (1997) showed that the non-selective mGlu receptor agonist ACPD increases sAPPα release from cultured astrocytes. However, no further studies aimed to explore the specific receptor involved or the mechanisms mediating that action. In this sense, a possible participation of adenylate cyclase-negatively coupled mGlu receptors (mGlu2,3,4,6,7 or 8) in the effect of ACPD had been suggested by the fact that cAMP and forskolin blocked ACPD-induced sAPPα production (Lee and Wurtman, 1997). Also, Efthimiopoulos et al. (1996) demonstrated that augments of intracellular cAMP levels inhibited sAPPα secretion from the C6 glial cell line. Indeed, a significant increase of cAMP levels in cerebrospinal fluid from AD patients was reported (Martinez et al., 1999). However, there is no direct evidence of involvement of group II mGlu receptors in APP shedding. With the aim of elucidating this issue, we used the selective, potent mGlu2/3 receptor agonist LY379268 which should only activate the mGlu3 receptor in astrocytes provided that this cell type does not express mGlu2 receptor. In fact, astrocytes have been shown to express mainly mGlu3 and mGlu5 receptors (Balazs et al., 1997; Condorelli et al., 1997; Ferraguti et al., 2001; Schools and Kimelberg, 1999) whereas neither mRNA nor protein for mGlu2 receptor has been found in this cell type. Also, several *in vivo* studies showed mGlu3 but no mGlu2 receptor expression in astrocytes (Mudo et al., 2007; Ohishi et al., 1993a, 1993b, 1998; Tamaru et al., 2001). Concordantly, Caraci et al. (2011a) demonstrated that neuroprotective actions of LY379268 in astrocytes are exclusively mediated by the mGlu3 subtype.

Also, like other authors in previous reports (Bandyopadhyay et al., 2006; Kieseier et al., 2003), we found basal expression of the main CNS α-secretases ADAM10 and ADAM17 in cultured rat astrocytes. Regarding BACE1 expression in astrocytes, contradictory results have been reported. Whereas in neurons the amount of Aβ produced is related to BACE1 expression levels, in astrocytes a poor correlation between BACE1 transcript and β-secretase activity was found (Bigl et al., 2000; Rossner et al., 2001). The current view is that astrocytes do not express BACE1 unless they are activated or cultured (Hartlage-Rubsamen et al., 2003; Rossner et al., 2001). In this line, we also now found basal expression of both mRNA and protein of the β-secretase BACE1 in cultured astrocytes. In the present study we also demonstrated that LY379268 significantly increased mRNA and protein levels of both ADAM10 and ADAM17 in astrocytes, which is correlated with induction of sAPPα release. On the contrary, BACE1 protein levels were strongly downregulated by LY379268. All together, these results indicate that the anti-amyloidogenic action of mGlu3 receptor rests on two pillars: stimulation of the α-secretase-mediated pathway and repression of the β-secretase-mediated amyloidogenic pathway, thereby resulting in inhibition of neurotoxic Aβ overproduction. Nevertheless, mRNA levels of BACE1 were increased by LY379268, an effect which is not in agreement with the marked down-regulation of BACE1 protein caused by the mGlu3 receptor agonist. This lack of correlation was previously reported in AD brains (Gatta et al., 2002; Marcinkiewicz and Seidah, 2000; Preece et al., 2003). It was also

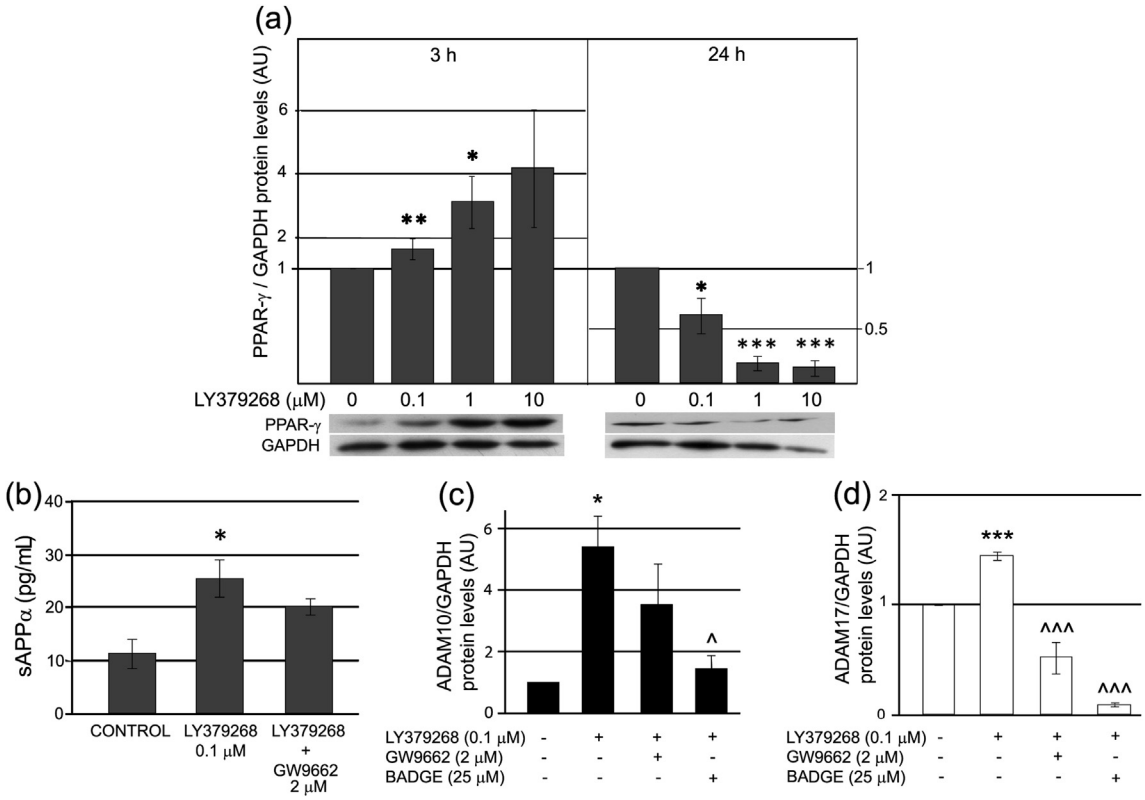

Fig. 4. Involvement of PPAR-γ in the effect of LY379268. (a) Cultured astrocytes were incubated with LY379268 (0.1–10 μM) in serum-free MEM for 3 or 24 h, and total protein homogenates were assayed by Western blot for PPAR-γ using GAPDH as endogenous control. OD of revealed bands was measured and values normalized to GAPDH and expressed as arbitrary units (AU) relative to control group ($n = 3$–4). (b) Cultured astrocytes were incubated with LY379268 (0.1 μM) in the presence or absence of GW9662 (2 μM) in serum-free MEM for 3 h. sAPPα levels in the culture medium were determined by solid phase sandwich ELISA and expressed as pg/mL. Each point represents the mean ± SEM of 5 technical replicates from one representative experiment. (c and d) Cultured astrocytes were incubated with LY379268 (0.1 μM) in the presence or absence of two different PPAR-γ antagonists, GW9662 (2 μM) and BADGE (25 μM) for 24 h, and total protein homogenates were assayed by Western blot for ADAM10 or ADAM17. OD values were normalized to GAPDH and expressed as AU relative to control group ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control group, $^{\wedge}p < 0.05$, $^{\wedge}p < 0.001$ versus LY379268.

exhaustively analyzed by De Pietri Tonelli et al. (2004), who described a regulatory mechanism of BACE1 translation carried out by a long BACE1 transcript leader whose 5′ non-coding region could silence BACE1 translation. In fact, it was seen that the human brain only expresses the long form of the transcript (De Pietri Tonelli et al., 2004; Mihailovich et al., 2007) and Bettegazzi et al. (2011) suggested that BACE1 expression is blocked at the translational level in cultured astrocytes. In our RTqPCR experiments, we may be amplifying the long (translation-silencer) form of BACE1 transcript, which increases with LY379268, thus leading to suppression of BACE protein levels.

It is now well known that sAPPα plays a role in the modulation of neuronal excitability, synaptic plasticity, neurite outgrowth, synaptogenesis, and cell survival (Chasseigneaux and Allinquant, 2012; Mattson, 1997; Turner et al., 2003). It induces neurite outgrowth in neural stem cell-derived neurons (Gakhar-Koppole et al., 2008) and also has proliferative and trophic actions in several cell types (Demars et al., 2011; Ohsawa et al., 1999; Pietrzik et al., 1998; Saitoh et al., 1989). The sAPPα peptide is composed of up to 6 domains, domains D1 and D6a likely being those that carry intrinsic neuroprotective activity, since they presented beneficial actions *per se in vitro* (Jin et al., 1994; Ohsawa et al., 1997; Qiu et al., 1995) and improved motor and cognitive outcome in a rat model of traumatic brain injury (Corrigan et al., 2011). Moreover, sAPPα protects cerebral cells against a variety of insults, including Aβ toxicity (Stein and Johnson, 2003). Even more interesting is the fact that sAPPα has memory-enhancing effects in certain behavioral paradigms (Bour et al., 2004). Central administration of sAPPα had potent memory-enhancing effects besides blocking learning deficits induced by scopolamine in mice (Meziane et al., 1998). Putting these facts together, not only the inhibition of β-secretase activity but also the induction of sAPPα release by LY379268 in astrocytes might equally account for the neuroprotection exerted by astroglial mGlu3 receptor activation described by Caraci et al. (2011a). These authors demonstrated that selective activation of mGlu3 receptor present in astrocytes prevented Aβ-induced neuronal death (Caraci et al., 2011a) and proposed that transforming growth factor β (TGF-β) could mediate the protective effect of these receptors. Since dual actions of TGF-β have been reported in AD (Caraci et al., 2011b; Luedecking et al., 2000; Tesseur et al., 2006; Wyss-Coray et al., 1997), we suggest that sAPPα may likewise be a good candidate for mediating neuroprotective effects of mGlu3 receptors against Aβ-related neurodegeneration.

In order to find possible mechanisms underlying the anti-amyloidogenic role of mGlu3 receptors, we explored the involvement of the nuclear receptor PPAR-γ since previous data indicate its participation in sAPPα production (Li et al., 2011), BACE1 transcription (Heneka et al., 2005; Li et al., 2011; Sastre et al., 2006; Zhao et al., 2011), Aβ accumulation (Camacho et al., 2004; Heneka et al., 2005; Sastre et al., 2003; Zhao et al., 2011) and even in Aβ clearance (Camacho et al., 2004; Mandrekar-Colucci et al., 2012). However, involvement of PPAR-γ in these events is highly dependent on the experimental model. To our knowledge, ours is the first report showing that mGlu receptors can induce PPAR-γ expression.

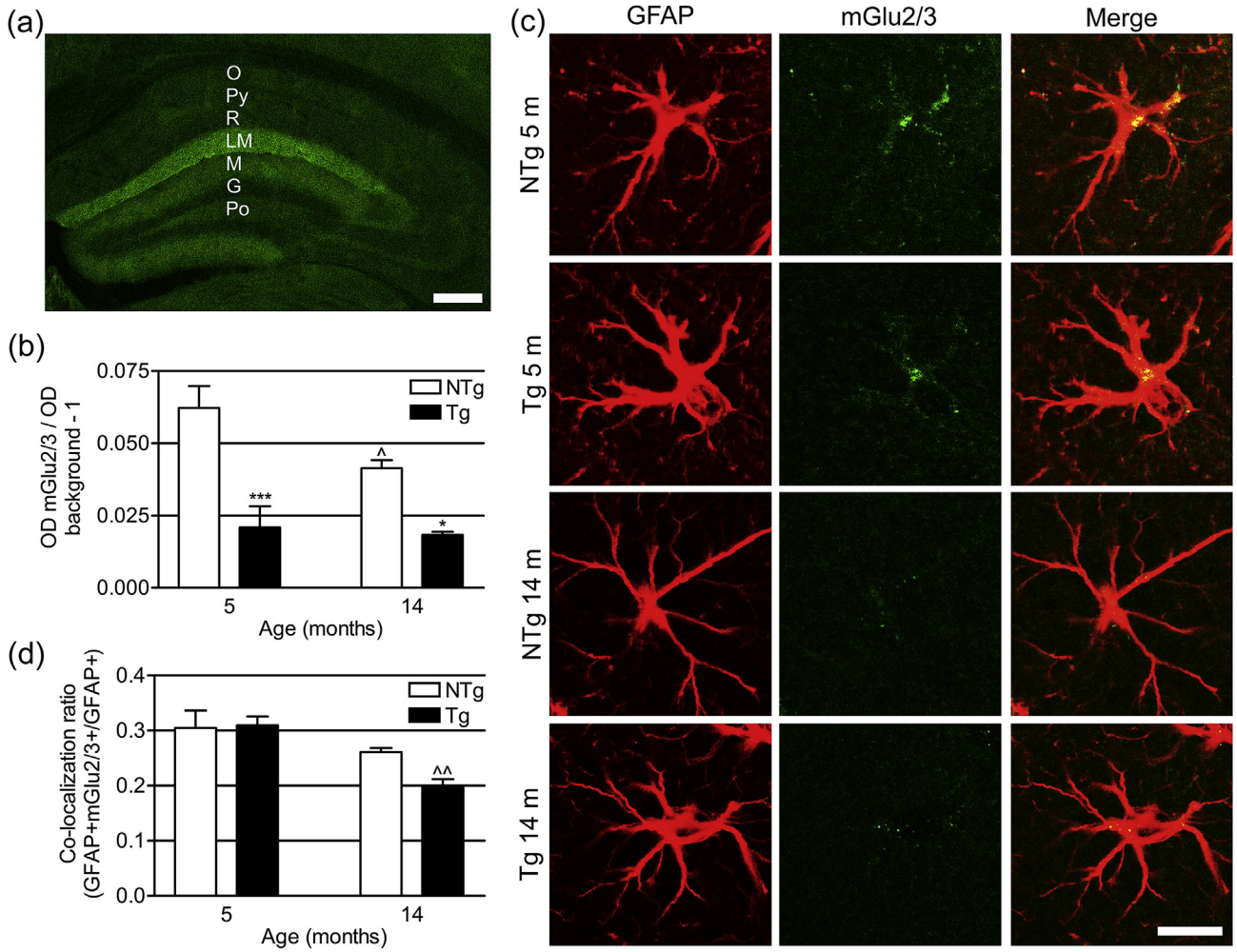

Fig. 5. Hippocampal mGlu2/3 receptor expression was down-regulated in transgenic PDAPP-J20 mice. (a) Total mGlu2/3 receptor immunostaining in the hippocampus clearly shows that mGlu2/3 receptors are mainly expressed in the stratum lacunosum moleculare of CA1. O = stratum oriens, Py = stratum pyramidale, R = stratum radiatum, LM = stratum lacunosum moleculare, M = stratum moleculare, G = stratum granulosum, Po = polymorphic layer. Bar = 250 μm. (b) Hippocampal slices from PDAPP-J20 transgenic (Tg, black bars) or non-transgenic (NTg, white bars) mice (at 5 and 14 months of age) were analyzed for mGlu2/3 receptor expression by immunohistochemistry as described in Materials and Methods (6 sections per brain, 4 animals per group). Immunolabeling from stratum lacunosum moleculare of CA1 region was digitally analyzed and OD calculated and expressed as the proportional increase of specific staining compared to background by the formula [specific LMol OD/background OD − 1]. (c) Confocal microscope images of hippocampal CA1 stratum lacunosum moleculare stained with anti-mGlu2/3 receptor (green) and anti-GFAP (red) antibodies. A representative image per group is shown. Since astrocytes do not express mGlu2 receptor subtype, anti-mGlu2/3 antibody should recognize only mGlu3 receptor subtype when co-localizing with GFAP (yellow). Bar = 20 μm. (d) Double immunolabeling from (c) was digitally analyzed and OD calculated and expressed as the ratio of $mGlu3^+/GFAP^+$ double staining to total $GFAP^+$ staining (6 sections per brain, 4 animals per group). $^*p < 0.05$; $^{***}p < 0.001$ versus NTg mice; $^{\wedge}p < 0.05$; $^{\wedge}p < 0.01$ versus 5-month-old mice of the same genotype. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

This effect was only seen 3 h post-LY379268 exposure (which correlates with the main effect of LY379268 in inducing sAPPα release) but not at longer times. The late negative regulation of PPAR-γ protein expression might respond to an autoregulatory mechanism of control of PPAR-γ activity. In fact, PPAR-γ has a very short half-life (Fu et al., 2003; Waite et al., 2001) due to poly-ubiquitination in lysine residues and degradation by proteasome which is dependent on activation (Hauser et al., 2000). In this respect, Fu et al. (2003) reported a biphasic effect of TGF-β on PPAR-γ expression in human aortic smooth muscle cells, with rapid and transient (30 min to 2 h) stimulation of PPAR-γ expression and strong suppression of its levels at longer incubation times (6–24 h). In any case, early and transient induction of PPAR-γ expression by LY379268 may still be enough to allow induction of sAPPα production and ADAM expression in astrocytes, as presented here. Although PPAR-γ has also been shown to repress BACE1 transcription in neural, kidney and fibroblastic cell lines (Sastre et al., 2006), our results seem to indicate that LY379268-induced PPAR-γ expression is not directly related to BACE1 mRNA down-regulation in cultured astrocytes. Instead, an alternative mechanism of LY379268-induced BACE1 modulation might be acting at the translational level in astrocytes.

Current evidence regarding expression of group II mGlu receptors either in animal models of AD or in human AD brains is not clear enough and has proven to be highly dependent on the experimental model, brain region and detection method employed. For example, mGlu2 receptor protein incremented in CA1 and CA3 areas of AD hippocampus but no changes were observed in the dentate gyrus (Lee et al., 2004). On the other hand, mGlu3 receptor mRNA levels decreased in the Tg2576 mice cerebellar granular cell layer whereas it increased in the cerebellar molecular cell layer, as determined by *in situ* hybridization (Cha et al., 2001). Immunoreactivity for mGlu2/3 receptor showed no significant modification, although it did show a tendency to decrease in CA1 in Tg 15-month-

old mice (Cha et al., 2001). In Tg PS2APP mice hippocampus [^{3}H] LY354740 binding decreased with age mainly in the dentate gyrus, lacunosum moleculare and subiculum (Richards et al., 2010) although these differences were absent when protein levels were assessed. Finally, Dewar et al. (1991) showed that glutamate metabotropic binding site levels were markedly reduced in the subiculum and in CA1 in subjects with AD. Our present results indicate that total mGlu2/3 protein levels in CA1 LMol were down-regulated in Tg PDAPP mice, whereas an age component was also significant. On the other hand, although a few descriptive studies have shown glial expression of group II mGlu receptors in the hippocampus (Petralia et al., 1996; Shigemoto et al., 1997), no reports discriminated glial mGlu3 receptor expression in AD hippocampi. Therefore, we determined glial expression of these receptors in our animal model and found a clear GFAP$^+$-astroglial localization of mGlu3 receptor. Specifically in astrocytes, anti-mGlu2/3 receptor antibody should bind to mGlu3 subtype as this is the only group II mGlu receptor subtype present in these cells, as stated above. Here, we described for the first time a reduced co-localization between mGlu3 receptor and GFAP$^+$-cells in 14-month-old Tg mice compared to 5-month-old Tg mice and to 14-month-old NTg mice, whereas minor differences were observed between 14-month-old versus 5-month-old NTg mice. Therefore, these reported differences suggest that: i) alteration in astrocytic mGlu3 receptor levels in Tg mice might partly account for the overall decrease in this receptor expression in the whole CA1 LMol; and ii) it is possible that neurodegenerative alterations in PDAPP mice are related to glial dysfunction that includes down-regulation of mGlu3 receptor levels in the hippocampus, since astroglial mGlu3 receptors might play an anti-amyloidogenic role as we have now proposed. In fact, we have corroborated in our PDAPP colony that amyloid plaque load in CA1 subfield significantly increases in 8-month-old and older Tg mice versus 5-month-old Tg mice (Beauquis et al., 2013) as it was already reported for this and other AD murine models (Galvan et al., 2006; Oddo et al., 2003; Wright et al., 2013).

In conclusion, the present study establishes a precedent for therapeutic implications involving mGlu3 receptors, α-secretases, sAPPα, PPAR-γ activation, and astroglial cells as key players in AD pathology. While it is clear that Aβ plays a crucial role in the pathogenesis of AD, the actual efficiency of targeting Aβ for mid-to-late stage AD intervention is now being questioned (Lane et al., 2012). In this context, the major relevance of our findings lies in the ability of astroglial mGlu3 receptors to affect multiple factors of AD etiology. We demonstrated that activation of astroglial mGlu3 receptors increased α-secretase expression whereas it decreased BACE1 levels, thereby promoting the non-amyloidogenic pathway and neurotrophic sAPPα production, simultaneously precluding the amyloidogenic processing of APP. mGlu3 receptor activation can also increase PPAR-γ levels, which might not only mediate sAPPα production but it is also known to induce glial Aβ uptake and degradation. Also, astrocytes - through mGlu3 receptor activity – are known to modulate several downstream events in AD progression such as glutamate excitotoxicity (Corti et al., 2007; Kingston et al., 1999; Yao et al., 2005; Zhong et al., 2005; Zhou et al., 2006), inflammation (Durand et al., 2010; Zhou et al., 2006) and oxidative stress (Berent-Spillson et al., 2004; Durand et al., 2010), and to improve neurotrophic factor production (Bruno et al., 1998; Ciccarelli et al., 1999; D'Onofrio et al., 2001). Therefore, astroglial mGlu3 receptors could be important candidates for comprehensive AD therapy. However, the fact that the effects of mGlu3 receptor agonists in microglia (Taylor et al., 2002) or neurons (Kim et al., 2010) are not identical to those in astrocytes suggests the need to develop therapeutic strategies based on the selective activation of these receptors in astroglial cells.

5. Disclosure statement

There are no current or potential conflicts of interest for any author or any of the authors' institutions.

Acknowledgments

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