

DISORDERS OF THE NERVOUS SYSTEM

Amyloid β inhibits retinoic acid synthesis exacerbating Alzheimer disease pathology which can be attenuated by an retinoic acid receptor α agonist

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Abstract

The retinoic acid receptor (RAR) α system plays a key role in the adult brain, participating in the homeostatic control of synaptic plasticity, essential for memory function. Here we show that RAR α signalling is down-regulated by amyloid beta (A β), which inhibits the synthesis of the endogenous ligand, retinoic acid (RA). This results in the counteraction of a variety of RAR α -activated pathways that are key in the aetiopathology of Alzheimer's disease (AD) but which can be reversed by an RAR α agonist. RAR α signalling improves cognition in the Tg2576 mice, it has an anti-inflammatory effect and promotes A β clearance by increasing insulin degrading enzyme and neprilysin activity in both microglia and neurons. In addition, RAR α signalling prevents tau phosphorylation. Therefore, stimulation of the RAR α signalling pathway using a synthetic agonist, by both clearing A β and counteracting some of its toxic effects, offers therapeutic potential for the treatment of AD.

Introduction

Alzheimer's disease (AD) has two major neuropathological hallmarks, the accumulation of amyloid beta (A β) in extracellular plaques and the presence of intracellular neurofibrillary tangles, whose main composition is hyperphosphorylated tau (Serrano-Pozo *et al.*, 2011). The A β hypothesis (Hardy & Selkoe, 2002) about the cause of AD has prompted the search for treatments which clear the excess A β from the brain, as there is some evidence that the cause of late-onset AD is inadequate clearance, rather than excess production (Mawuenyega *et al.*, 2010). Specific antibodies to A β have been a favoured approach (Delrieu *et al.*, 2012), but recently a drug which penetrates the blood–brain barrier and elevates the level of apolipoprotein E (apoE), which acts as part of the physiological A β clearance mechanisms, has been suggested (Cramer *et al.*, 2012). According to the most recent version of the A β hypothesis, the various pathogenic features of AD are primarily caused by soluble oligomeric forms of A β , particularly of its 42 amino-acid form, acting either extracellularly or intracellularly (LaFerla *et al.*, 2007; Jin *et al.*, 2011).

Microglia play a major role in A β clearance and they surround the A β plaques which induce a phagocytic response (Tuppo & Arias, 2005). They can also synthesize the A β -clearing enzymes insulin degrading enzyme (IDE) (Tuppo & Arias, 2005) and neprilysin (NEP) (Marr *et al.*, 2004). However, inflammatory processes, caused by 'activated' microglia, are involved in the pathogenesis of AD (Tu-

ppo & Arias, 2005; Rogers *et al.*, 2007). One hallmark of this process is the secretion of the pro-inflammatory cytokine tumour necrosis factor (TNF) α , which may lead to further degeneration (Smith *et al.*, 2012) and prevention of A β clearance (Hickman *et al.*, 2008). In addition, the neurons themselves can remove the toxic oligomers of A β via the activation of IDE and NEP (Vekrellis *et al.*, 2000; Carson & Turner, 2002). Therefore, an ideal therapeutic agent would be one that prevents the 'activation' of microglia as well as stimulating the IDE and NEP pathways in both microglia and neurons.

Although the aetiology of non-familial AD remains unclear, previous work has suggested that a deficit in retinoic acid (RA) signalling, which is essential for normal brain maintenance (Maden, 2007), may be associated with AD (Goodman & Pardee, 2003; Corcoran *et al.*, 2004; Ding *et al.*, 2008; Tippmann *et al.*, 2009; Donmez *et al.*, 2010; Jarvis *et al.*, 2010). It has not yet been shown how a deficit in RA signalling could occur. Given the recently documented inverse correlation between intracellular calcium concentration and RA synthesis (Wang *et al.*, 2011) and the role of A β in increasing intracellular calcium (Camandola & Mattson, 2011; Ferrizzi & Carafoli, 2011), we have investigated whether A β can prevent RA synthesis in the adult brain.

In earlier work, we showed that agonists of the retinoic acid receptor (RAR) α , but not of the RAR β or γ , were strikingly effective in lowering A β levels, having an especially strong action on intracellular A β 42 (Jarvis *et al.*, 2010). The mechanism was presumed to be explained by a reduction of A β production, due to the observed increase in activity of an alpha secretase, ADAM10, thus favouring the non-amyloidogenic processing of amyloid precursor

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protein (APP) over its amyloidogenic cleavage. However, given the dramatic decrease in extracellular soluble A β in Tg2576 mice as a response to RAR α signalling, which does not alter APP processing (Jarvis *et al.*, 2010), we have examined here whether there was an additional effect of RAR α activity on the clearance of A β .

Our data suggest that A β can prevent the synthesis of RA in the adult brain, including the microglia. This is associated with the loss of activity of both NEP and IDE, an increase in the secretion of the inflammatory cytokine TNF α and the phosphorylation of tau. All of these processes can be reversed by an RAR α agonist and the accumulation of A β in extracellular plaques reduced. In addition, we show that the cognitive behavioural deficits which develop in the Tg2576 mouse can be reversed.

Materials and methods

Animal treatments and cognitive behavioural studies

All procedures were performed in accordance with the Animal Scientific Procedures Act (1986), UK. Local ethics approval was obtained from King's College London in accordance with European Communities Council Directive of 24 November 1986. Tg2576 mice on a 129S6 background, APPSWE/tau(P301L) and respective wild-type (wt) littermate mice were purchased from Taconic Farms (Germantown, NY, USA). Mice were maintained on a 12/12-h light–dark cycle at 20–22 °C and given food and water *ad libitum*. Mice ($n = 7–8$ per group) were treated by intraperitoneal injections (i.p.) of either vehicle [80% dimethyl sulfoxide (DMSO) in distilled water] or 1 mg/kg with retinoids three times a week from 15 to 18 months of age for the Tg2576 mice and from 12 to 15 months age for the P301L mice. In addition the P301L mice were treated with 10 mg/kg of lithium (Sigma Aldrich, Dorset, UK) for the same amount of time as the retinoid treatment. These doses were based on previous work (Jarvis *et al.*, 2010). The retinoids used were RAR α selective, AM 580; RAR β selective, CD2019; or RAR γ selective, CD437 (Jarvis *et al.*, 2010). All the retinoid agonists were synthesized by Sygnature Chemical Services, Nottingham, UK.

T-maze spontaneous alternation

This was carried out as previously described (Deacon & Rawlins, 2006) during the last 3 weeks of the retinoid treatment. Two trials were carried out separated by 30 min per group of mice ($n = 7–8$), and repeated on a weekly basis for a total of six trials. The mouse was placed at the end of the start arm facing away from the goal arms and allowed to freely explore the maze. Once the mouse tail tip was fully within one of the goal arms, the goal arm door was closed and the mouse allowed to explore for 30 s. The central partition and goal arm door were then removed and the mouse again placed in the start arm as above, and allowed free choice to enter either of the goal arms. Once the mouse tail tip was fully within one of the arms the trial was terminated and the correct number of alternations was recorded. If the mouse did not enter a goal arm after 90 s the trial was ended.

Nest building

This was carried out as previously described (Deacon, 2006) in the last week of retinoid treatment. Mice ($n = 7–8$) were put in individual cages 2 h before dark. A nestlet (5 × 5 cm of cotton batting, Ancare; UK agent Lillico) was placed in the cage and the nest quality assessed the next day. The following scoring was used by an

observer blinded to the treatment: (i) nestlet not noticeably touched; (ii) 50–90% nestlet intact; (iii) 50–90% nestlet shredded but no identifiable nest; (iv) 90% nestlet is torn and a flat identifiable nest and the walls higher than the mouse at less than 50% of its circumference; and (v) more than 90% nestlet is torn and the nest walls higher than the mouse for 50% of its circumference.

Neuronal cultures

Cortical neurons were isolated from E15 mouse (NIH Swiss, Harlan, Oxon, UK). The brains were removed from the embryos and washed three times in PBS-1.5% glucose. Cortices without their meninges were then triturated through a 21G needle in the presence of PBS-1.5% glucose. The dissociated cells were then left for 5 min on ice to allow debris to settle. The supernatant was transferred to a 15 mL falcon tube and spun for 5 min at 134 g. Cells were plated at a density 0.5×10^6 per well on 13-mm² glass coverslips in 24-well plates (Nunc, Invitrogen, Paisley, UK) precoated with 10 μ g/mL poly-D-lysine (Sigma Aldrich, Dorset, UK). The neurons were grown in Neurobasal media (Invitrogen), supplemented with B-27 (Invitrogen), 2 mM glutamine, 1.5% glucose, 100 μ g/mL streptomycin and 60 μ g/mL penicillin (Invitrogen), at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cultures were used after 7 days and were greater than 98% neurons as judged by β -tubulin III staining. All the cultures used were similar in appearance at the time of treatment. The retinoids were made up at 1000 × stock concentrations in DMSO. A β 42 (California Peptide Research Inc., Napa, CA, USA) was made up at 1000 × concentration in DMSO. Cortical neurons were cultured for 7 days in serum-free medium and then for 3 days with 1 μ M human A β 42 and either 0.1 μ M of AM 580 alone or in the presence of A β . Culture conditions were three wells per treatment carried out three times.

Microglia cultures

Primary mixed glial cultures were prepared as described previously (McCarthy & de Vellis, 1980) using a modified protocol. Briefly, mixed glial cultures were obtained from the cortices of postnatal mice (P5–P8). Cultures were maintained at 37 °C (5% CO₂/95% O₂) in medium containing 15% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Sigma Aldrich) for 10–14 days. Microglial cells were then harvested by forceful shaking for 1 min by hand and plated on poly-D-lysine-coated glass coverslips or plastic six-well plates. After 3 days, microglia cultures were treated for 3 days, with either vehicle or A β 42 (0.02 μ M), or AM 580 (0.1 μ M) with or without A β 42.

Antibodies

The following antibodies were used: rabbit polyclonal anti-IDE (1 : 100, Abcam plc, Cambridge, UK), rabbit polyclonal anti-RAR α (1 : 100, Santa Cruz Biotechnology, Heidelberg, Germany), mouse monoclonal anti-beta amyloid (1 : 100 6E10, Covance, Emeryville, CA, USA), anti-goat NEP (1 : 50, R&D Systems, Minneapolis, MN, USA), rabbit polyclonal anti-ionized calcium binding adaptor molecule 1 (Iba1, 1 : 1000, Wako Chemicals USA, Inc.), goat anti-Iba1 (1 : 200, Abcam), rabbit polyclonal anti-apoE [1 : 100 and 1 : 1000 (for immunohistochemistry and Western Blotting, respectively), Abcam]. Secondary antibodies were AlexaFluor™ 594 and AlexaFluor™ 488 (1 : 1000, Molecular Probes, Invitrogen) and AlexaFluor™ 680 (1 : 5000, Molecular Probes, Invitrogen). DAPI was used to stain nuclei (1 μ g/mL, Sigma Aldrich).

Immunohistochemistry

Neuronal cultures

Immunohistochemistry was carried out as previously described (Goncalves *et al.*, 2005). Neuronal cultures were washed with PBS for 1 min. They were then fixed in 4% paraformaldehyde for 30 min and washed three times for 5 min each in PBS-0.02% Tween. They were incubated in primary antibody in PBS-0.02% Tween at 4 °C overnight. Primary antibody was removed by washing three times for 5 min each in PBS-0.02% Tween. They were incubated in the secondary antibody for 1 h at room temperature in PBS-0.02% Tween, and then washed in PBS three times for 5 min. The coverslips were then mounted using FluoroSave™ reagent (Merck, UK). Incubation of neurons with secondary antibodies in the absence of primary antibodies produced a very weak diffuse staining of cell bodies that did not overlap with the primary antibody-specific staining (data not shown).

Brain sections

Tg2576 and 129S2/SvHsd mice were deeply anaesthetized by i.p. injection of pentobarbitone, and transcardially perfused with heparinized saline. Brains were rapidly removed and longitudinally bisected. Half hemispheres were post-fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer) for at least 2 days at room temperature. Tissue was then embedded in paraffin wax and 5- μ m sagittal sections cut throughout each block. Sets of consecutive sections, randomly chosen at four different levels per brain, were used for immunostaining. Sections were first dewaxed in xylene and 100% industrial methylated spirits, then heated in citric acid (10 mM, pH = 6), until boiling, then washed under a running tap for 5 min. Sections were then blocked with 1% BSA for 15 min, followed by overnight incubation at 4 °C with the primary antibody. For 6E10 staining, sections were incubated for 20 min in 70% formic acid before overnight incubation with primary antibody. After three 5-min washes with PBS-0.02% Tween, sections were incubated with the corresponding fluorescent secondary antibody. The slides were then mounted using FluoroSave™ reagent (Merck).

Microscopy

Multichannel fluorescence (DAPI-FITC-Texas Red filter set) images were captured using a Zeiss LSM 700 laser-scanning confocal microscope, with a 63 \times oil-immersion Aplanachromat objective (Carl Zeiss). Settings for gain, aperture, contrast and brightness were optimized initially, and held constant throughout each study so that all sections were digitized under the same conditions of illumination. Channels were imaged sequentially to eliminate bleed-through and multichannel image overlays were obtained using Adobe Photoshop 7.0 (Adobe Systems).

F9-RARE LacZ reporter assay

Murine F9 embryonal carcinoma cells which stably express an RAR β 2-promoter construct were used as previously described (Sonneveld *et al.*, 1999). Cells were scored on a blue vs. not blue basis and number or percentage of LacZ-positive cells were quantified from five random fields of view from three independent experiments (Sonneveld *et al.*, 1999).

ELISAs and enzyme activity assays

Quantification of IDE activity was done using the Calbiochem® InnoZyme™ Insulysin/IDE Immunocapture Activity Assay Kit, following the manufacturer's instructions.

NEP activity assay was done following previous methods (Tamboli *et al.*, 2010). NEP substrate was *N*-succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin (Sigma Aldrich) and NEP inhibitor thiorphan (Sigma Aldrich).

For TNF α and A β 42 quantification, a mouse TNF α ELISA Kit (Molecular Probes, Invitrogen) and a human A β 42 kit (Molecular Probes, Invitrogen) were used, respectively, following the manufacturers' instructions.

All results are a combination of three independent experiments and all samples have been normalized for protein concentration.

Production of A β 42 oligomers

A β 42 monomers and oligomers were prepared as previously described (Ryan *et al.*, 2010). Briefly, human synthetic A β 42 (California Peptide Research, Napa, CA, USA) was suspended in chilled 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP; Sigma-Aldrich) to 1 mM. After vortexing, the A β /HFIP solution was aliquoted into microfuge tubes, lyophilized in a Speed-Vac and stored desiccated at -20 °C until required. A β oligomers were prepared by resuspending the desiccated peptide to 5 mM in anhydrous dimethyl sulfoxide (DMSO). Following a 10-min bath sonication, the suspension was diluted to 100 μ M by cold PBS + 0.05% SDS, vortexed for 30 s and stored at 4 °C for 24 h to allow oligomer formation. The solution was then diluted to 50 μ g/mL (11 μ M) with PBS and added to neuronal cultures or incubated at 4 °C to allow further aggregation. A β monomers were prepared immediately before use. Desiccated peptide was resuspended to 5 mM in DMSO, as previously described, and further diluted to 50 μ g/mL with PBS. The suspension was vortexed briefly and added to cell cultures.

Quantification of A β oligomers in neuronal cultures

Amyloid beta oligomers were quantified in neuronal cultures as previously described (Cheng *et al.*, 2009). Four images per culture condition were captured, and A β oligomers were quantified manually in five random fields, by an operator blinded to treatments. The experiment was repeated three times.

Western blotting

Protein was isolated from the neuronal and microglial cultures in a lysis solution containing 20 mM Tris-HCl (pH 7.6), 1% NP-40 (Sigma Aldrich), 137 mM NaCl and 2 mM EDTA, with 1 mM phenylmethylsulfonyl fluoride, in the presence of a protease inhibitor mixture (Sigma Aldrich). Cell lysates were centrifuged for 10 min at 9000 *g*. The protein concentration in the supernatants was determined with the BCA Protein Assay (Pierce, Rockford, IL, USA). Ten micrograms of protein was loaded onto 10% SDS-PAGE gels. Semi-dry blotting was performed, and the blots were probed with appropriate antibodies. The cell lysates were then incubated with the secondary antibody and visualized with an Odyssey infrared scanning system. For a loading control, the blots were probed with mouse anti-actin and developed as above. Signal density was calculated as the ratio of signal intensity to β -actin.

Data analysis

Data were analysed using either one-way ANOVA followed by Tukey's or Fisher's test, or for Tmaze one-way repeated-measures ANOVA, followed by the Holm-Sidak test; or for nest building, one-way ANOVA on ranks, followed by Dunn's test using SIGMA STAT

software (SPSS Software Ltd, Birmingham, UK). Comparisons were made between appropriate groups and differences were considered statistically significant at the level of $P < 0.05$. Results are given as mean \pm SE or \pm SD and P -values are provided as summary statistics.

Results

RAR α signalling removes A β plaques and improves cognition in the Tg2576 mouse

Fifteen-month-old Tg2576 mice were treated with 1 mg/kg of either RAR α , β or γ selective agonists three times a week for 6–12 weeks and amyloid plaques were quantified in the cortex and hippocampus (Fig. 1A). In the 6-week treated mice, plaque load increased from 15 to 16.5 months by $\sim 60\%$ in vehicle-treated mice. In contrast, treatment with the RAR α agonist, AM 580, resulted in a net reduction by about 50% of the number of plaques 6 weeks earlier (Fig. 1B, one-way ANOVA followed by Tukey's test, $F_{2,6} = 10.109$, $P = 0.01$). This result suggests that it is unlikely that the sole effect of the AM 580 is reduced production of A β and that it also facilitates clearance. Similarly, in animals treated for 12 weeks (Fig. 1C), the animals treated with AM 580 showed a significant decrease in

plaque number compared with the vehicle-treated mice. Neither the RAR β agonist (CD2019) nor the RAR γ agonist (CD437) had any effect on plaque load, matching our earlier result (Jarvis *et al.*, 2010) that the reduction of A β levels is exclusively RAR α -mediated (Fig. 1C, Tg2576 vehicle vs. Tg2576 AM 580, one-way ANOVA followed by Tukey's test, $F_{3,44} = 10.101$, $P < 0.001$).

In parallel, the effect of each of the retinoid agonists on cognition was assessed by two tests, T-maze alternation and nest building (Deacon, 2006; Deacon & Rawlins, 2006). For these studies, age-matched wt littermates, treated with AM580, were included as another control group. The T-maze spontaneous alternation task was carried out during the last 3 weeks of retinoid treatment. The vehicle-treated Tg2576 mice made an average of 43% of successful trials compared with 72% for wt mice (Fig. 1D, one-way repeated-measures ANOVA, $P < 0.001$ followed by Holm-Sidak test, $F_{3,16} = 49.21$, $P < 0.001$). In the RAR β - or γ -treated Tg2576 mice there was no significant difference in the number of correct alternations compared with the vehicle-treated Tg2576 ones (Fig. 1D, one-way repeated-measures ANOVA as above all $P > 0.05$). However, in the RAR α agonist-treated Tg2576 mice the number of correct alternations increased significantly compared with the vehicle-treated Tg2576 mice (Fig. 1D, one-way repeated-measures ANOVA as above, $P < 0.001$) and was not significantly different from the vehicle-treated wt mice.

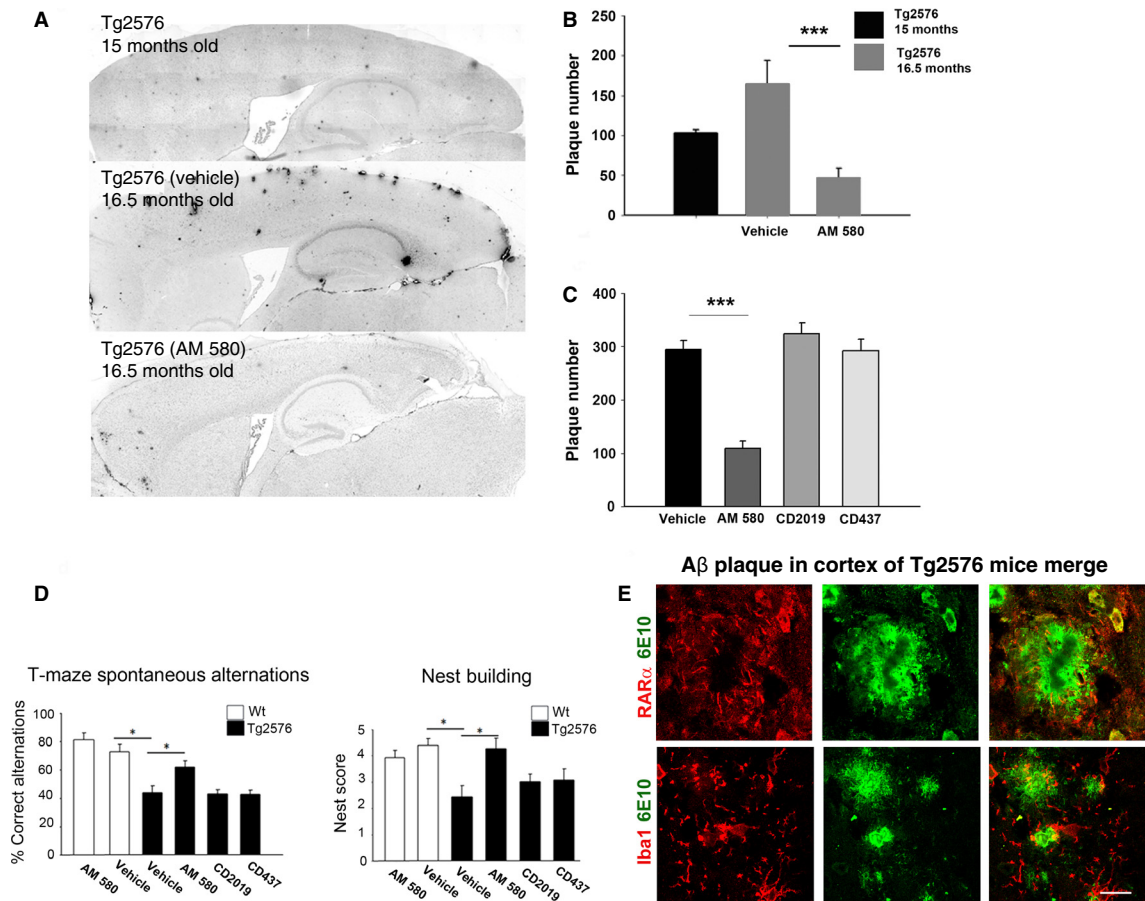


FIG. 1. AM 580 induces A β plaque clearance and improves cognition in Tg2576 mice. (A) Immunohistochemistry for 6E10 in brains of Tg2576 mice. (B) Six weeks of AM 580 (1 mg/kg, i.p., three times a week) treatment significantly reduces plaque load in cortex and hippocampus in 15-month-old Tg 2576 mice (Student's t -test, $***P < 0.001$). (C) Twelve weeks of treatment with AM 580 (1 mg/kg, i.p., three times a week) significantly reduced plaque load whereas CD2019 or CD437 (both 1 mg/kg, i.p., three times a week) had no effect (one-way ANOVA, followed by Tukey's test, $***P < 0.001$). (D) Performance in behaviour tests of wt and Tg2576 mice treated either with vehicle or with retinoid agonists, T-maze spontaneous alternations and nest building, $n = 7-8$. For T-maze one-way repeated-measures ANOVA, followed by Holm-Sidak test; $n = 7-8$, for nest building, one-way ANOVA on ranks, followed by Dunn's test, $n = 7-8$, error bars are SEM, $*P < 0.05$. (E) RAR α and microglia surround A β plaques in cortex of Tg2576 vehicle-treated mice. Scale bar = 20 μ m.

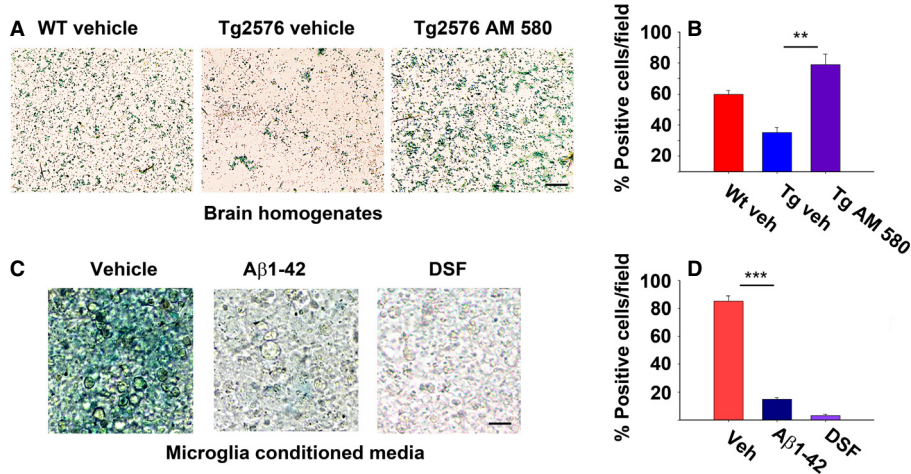


FIG. 2. AM 580 attenuates Aβ-induced decrease of RA synthesis. (A) X-gal staining of F9 cells showing RA synthesis in brain homogenates; scale bar = 150 μm. (B) Quantification of RA synthesis shows that AM 580 (1 mg/kg, i.p., three times a week for 3 months) completely restores Aβ-induced decrease in RA synthesis in Tg2576 mice brains (one-way ANOVA, followed by Tukey's test, $^{**}P < 0.01$). (C) X-gal staining in F9 cells showing RA synthesis from microglia cultures; scale bar = 50 μm. (D) Quantification of positive cells shows that Aβ1-42 (0.02 μM, 3 days) significantly blocks RA synthesis in microglia cultures (one-way ANOVA, followed by Tukey's test, $^{***}P < 0.001$); $n = 3$, for all experiments. Error bars are SEM.

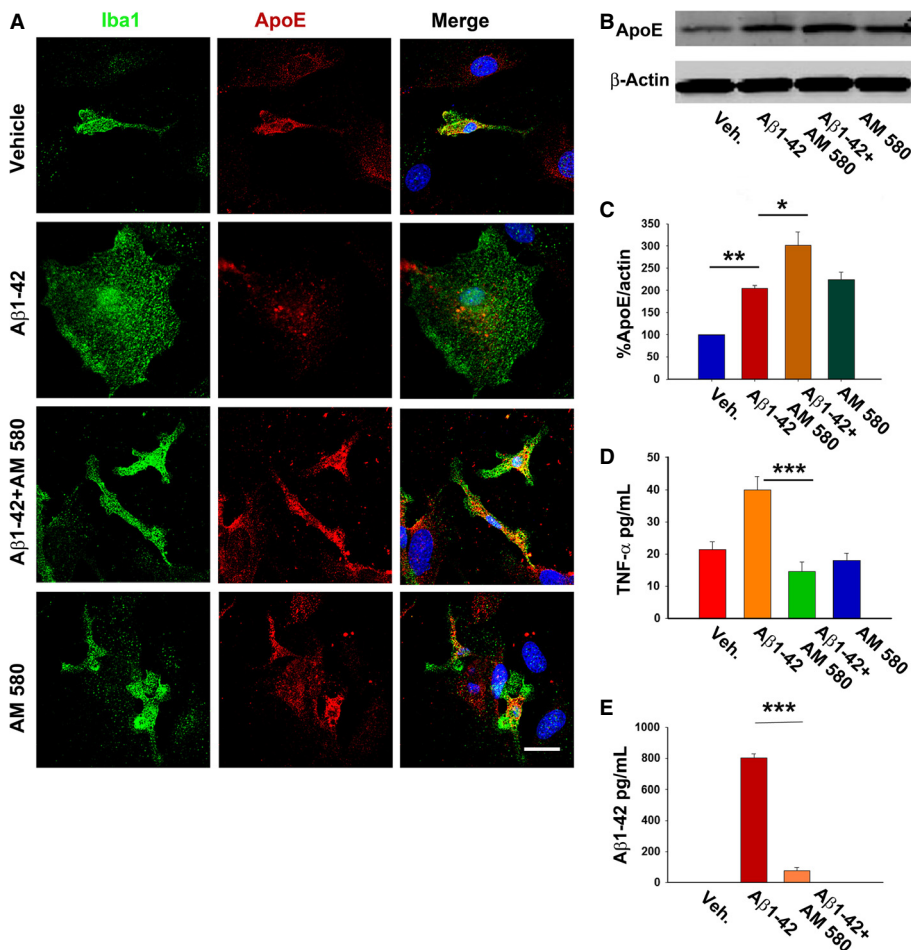


FIG. 3. AM 580 induces Aβ clearance in microglia via up-regulation of apoE and down-regulation of TNFα. (A) Expression of apoE in microglia cultures; scale bar = 50 μm. (B, C) Quantification of microglial apoE by Western blotting (one-way ANOVA, followed by Tukey's test, $^{**}P < 0.01$, $^{*}P < 0.05$). (D) Suppression of TNFα secretion by AM 580 (0.1 μM, 3 days) in Aβ- (0.02 μM, 3 days) treated microglia cultures measured by ELISA (one-way ANOVA, followed by Tukey's test, $^{***}P < 0.001$). (E) AM 580 (0.1 μM, 3 days) induces clearance of Aβ1-42 from media in Aβ1-42- (0.02 μM, 3 days) treated microglia cultures (Student's, t -test, $^{***}P < 0.001$).

In the nest building test, which was carried out in the last week of retinoid treatment, the vehicle-treated wt mice scored an average of 4.5/5, whilst the similarly treated Tg2576 mice scored 2.5/5 (Fig. 1D, one-way ANOVA on ranks, followed by Dunn's test, $P < 0.05$). In the AM 580-treated Tg2576 mice there was a significant improvement in nest building compared with vehicle-treated Tg2576 (Fig. 1D, one-way ANOVA on ranks as above, $P < 0.05$), which was comparable to the wt mice. By contrast, there was no improvement produced by the RAR β or γ agonist on nest building in the Tg2576 mice (Fig. 1D, one-way ANOVA on ranks as above, all $P > 0.05$). Treatment with AM 580 in wt mice did not cause any significant difference in either test, compared with the wt vehicle-treated group (Fig. 1D).

A β prevents RA synthesis

Microglial cells play an essential role in A β clearance (Cameron & Landreth, 2010), so we investigated whether the RAR α was expressed in microglia present in the vicinity of amyloid plaques. Immunostaining with the microglia marker Iba1 and with RAR α antibody, in cortices of Tg2576 mice, showed co-localization (data not shown) and both were found to be expressed around amyloid plaques, which were labelled with the marker 6E10 (Fig. 1E). As there is a failure of plaque clearance in the Tg2576 mice, despite the presence of some RAR α , we investigated whether it was a reduction in the production of the endogenous ligand, RA, or a problem downstream of the reti-

noid activation, which was responsible. To make a direct check on the synthesis of RA, we used a bioassay with a RARE-lacZ RA reporter cell line, F9 (Sonneveld *et al.*, 1999). F9 cells were treated with homogenates of whole brain hemispheres of either wt, vehicle-treated Tg2576 or AM 580-treated Tg2576 mice. After overnight incubation, F9 cells were stained for X-gal and the intensity of staining was used to quantify RA synthesis. There was a significant decrease in RA synthesis in vehicle-treated Tg2576 mice, as shown by the lack of positive blue cells compared with wt mice, and this was reversed by AM 580 treatment (Fig. 2A and B, one-way ANOVA followed by Tukey's test, $F_{2,6} = 21.295$, $P = 0.002$). To confirm that this effect was at least partly taking place in the microglia, F9 cells were treated with conditioned media taken from microglial cultures that were treated for 3 days with either vehicle, A β 42 or Disulfiram (DSF), which blocks RA synthesis (Lipsky *et al.*, 1997). After 1 day F9 cells were stained for X-gal, as above. Microglia were shown to synthesize RA, which was prevented in the presence of DSF or A β 42 (Fig. 2C and D, one-way ANOVA followed by Tukey's test, $F_{2,6} = 371.545$, $P < 0.001$).

RAR α signalling stimulates A β clearance by increasing NEP and IDE activity

We next looked at the effect of RAR α signalling on apoE expression, as it is well known that increased A β in the brain produces a corresponding increase in apoE (Rossello *et al.*, 2012), perhaps by direct transcriptional action (Maloney & Lahiri, 2011), but yet not

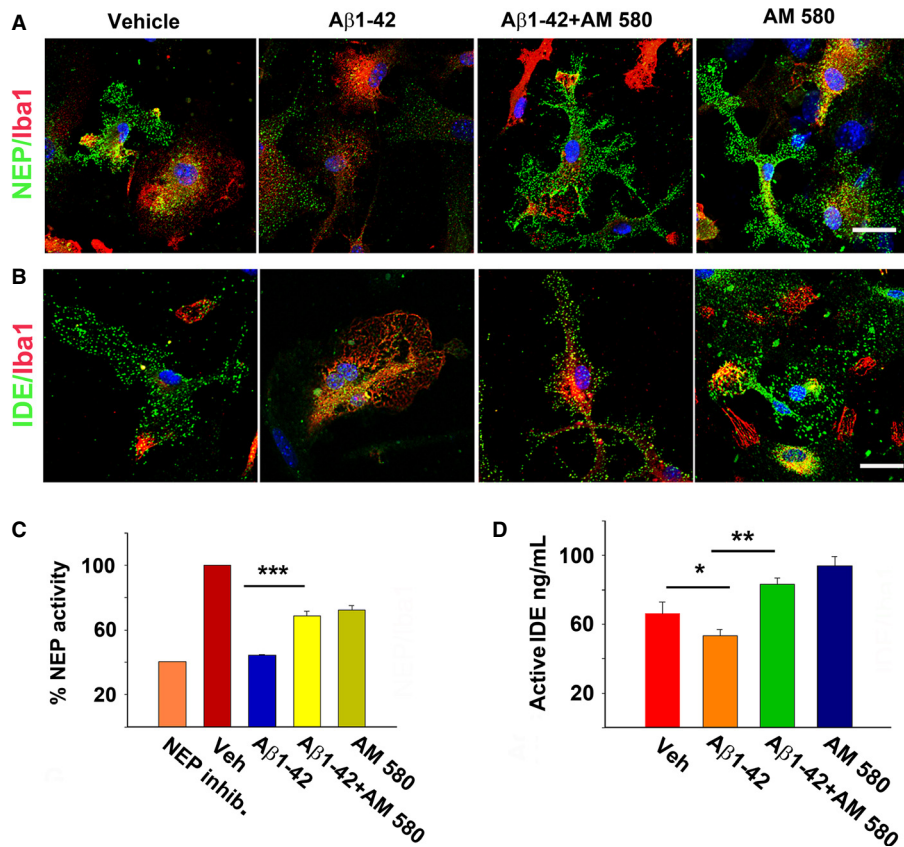


FIG. 4. AM 580 induces A β clearance in microglia by increasing activity of NEP and IDE. Expression of NEP (A) and IDE (B) in microglia cultures; scale bar = 20 μ m. (C) NEP activity in microglia cultures is significantly higher in A β 1-42-treated cultures treated concomitantly with AM 580 compared with A β 1-42-treated (one-way ANOVA, followed by Tukey's test, $***P < 0.001$). (D) IDE activity significantly increases in A β 1-42-treated microglia cultures with concomitant treatment of AM 580 compared with A β 1-42-treated (one-way ANOVA, followed by Fisher's test, $*P < 0.05$, $**P < 0.01$). $n = 3$ for all experiments, Error bars are SEM.

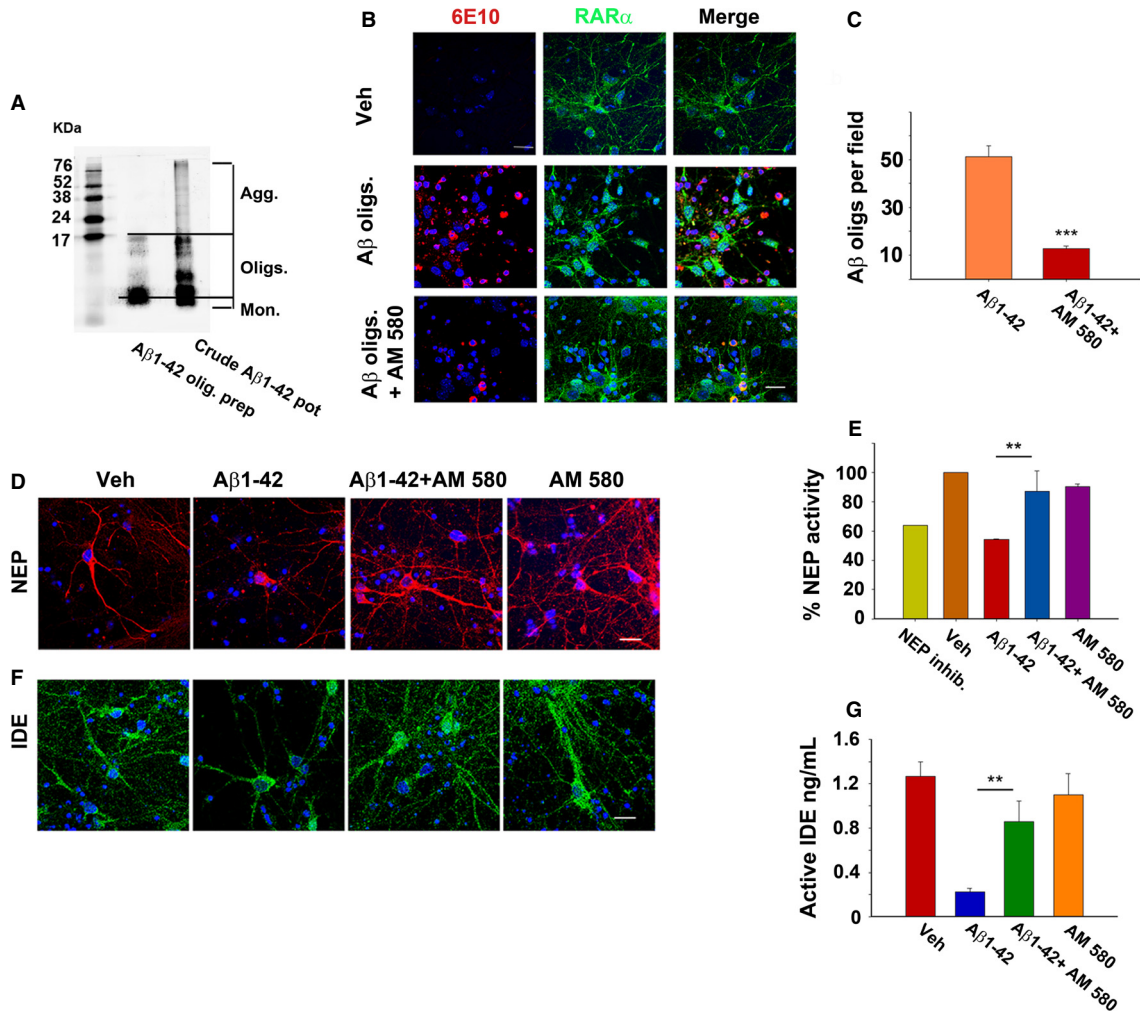


FIG. 5. AM 580 induces Aβ oligomeric clearance in neuronal cultures by increasing NEP and IDE activity. (A) Western blots of Aβ protein using 6E10 antibody, illustrating the formation of oligomeric forms of Aβ. (B) Immunostaining showing Aβ1-42 oligomers and RARα in cortical neuronal cultures; scale bar = 30 μm. (C) Quantification of oligomers shows significant decrease in the presence of AM 580 (Student's *t*-test, ****P* < 0.001). (D) Expression of NEP in neuronal cultures; scale bar = 20 μm. (E) NEP activity in membrane lysates is significantly increased in Aβ1-42- (1 μM, 3 days) treated cultures treated concomitantly with AM 580 (0.1 μM, 3 days) compared with Aβ1-42-treated (one-way ANOVA, followed by Fisher's test, ***P* = 0.001). (F) Expression of IDE in neuronal cultures; scale bar = 20 μm. (G) Active IDE in neurons is significantly increased in Aβ1-42-treated cultures treated concomitantly with AM 580 compared with Aβ1-42-treated (one-way ANOVA, followed by Fisher's test, ***P* = 0.002).

sufficient to induce effective clearance of Aβ (Cramer *et al.*, 2012). We confirmed that Aβ increases apoE in microglia cultures, and more importantly that when AM 580 was concomitantly added to the cultures, it significantly induced apoE expression compared with Aβ alone (Fig. 3A–C, one-way ANOVA followed by Tukey's test, $F_{3,8} = 33.151$, *P* = 0.03). We then approached downstream pathways involved in Aβ clearance.

It is well known that early microglial recruitment to the amyloid plaques promotes their clearance (Hickman *et al.*, 2008), but the subsequent release of inflammatory cytokines, including TNFα, leads to the downregulation of genes which are involved in the clearance of Aβ, such as IDE and NEP (Nalivaeva *et al.*, 2012). Therefore, we checked whether the RARα pathway had an anti-inflammatory action by downregulating TNFα secretion. Microglial cultures were treated with either vehicle or 20 nM Aβ42, in the presence or absence of AM 580, for 3 days. TNFα was then quantified in the media using a specific TNFα ELISA kit. We found that AM 580 significantly decreased TNFα release from Aβ-treated microglial cultures (Fig. 3D, one-way ANOVA followed by Tukey's test, $F_{3,8} = 14.446$, *P* < 0.001). To test the capability of RARα activa-

tion to induce Aβ clearance in Aβ42-treated microglial cultures, the amount of Aβ42 in the medium was assayed using a specific human Aβ42 ELISA kit. AM 580 significantly reduced the level of Aβ in the medium, by about 90%, compared with control cultures (Fig. 3E, Student's *t*-test, $t_{10} = 7.444$, *P* < 0.001). These results, taken together, clearly illustrate the connection between RARα signalling, modulation of TNFα production and Aβ clearance by the microglia.

Two of the major mechanisms by which microglia promote proteolytic Aβ clearance are through the action of NEP and IDE, with NEP being the sole documented mechanism for cleaving oligomeric Aβ (Nalivaeva *et al.*, 2012). The actions of these two enzymes are facilitated by the presence of apoE (Jiang *et al.*, 2008). We therefore checked the activity and location of these two enzymes in Aβ42-treated microglia, in the presence or absence of AM 580. Microglial cultures were treated as before, and double stained for both Iba1 and either NEP or IDE. Both proteolytic enzymes showed a decrease in expression in Aβ-treated microglia compared with vehicle-treated microglia, which was reversed by AM 580 treatment (Fig. 4A and B). Both enzymes were found to be expressed on the

microglial surface. As NEP acts as an ectoenzyme (Marr *et al.*, 2004), to test cell surface activity we used an NEP activity assay (Tamboli *et al.*, 2010), whereas IDE activity was measured in the culture medium, as microglial cells release substantial amounts of IDE (Qiu *et al.*, 1997). We found that A β caused a significant decrease in IDE activity and AM 580 reversed the A β -induced suppression of activity, to above vehicle levels for IDE and near to vehicle levels for NEP (Fig. 4C and D, for NEP, one-way ANOVA followed by Tukey's test, $F_{2,5} = 97.066$, $P < 0.001$; for IDE, one-way ANOVA followed by Fisher's test, $F_{3,9} = 12.846$, $P = 0.04$ for veh. vs. A β and $P = 0.01$ for A β vs. AM 580-treated cultures).

RAR α signalling removes A β oligomers

The degradation of A β plaques by cleaving enzymes could lead to a local increase in soluble oligomeric A β . Indeed, there is a local association between plaques and raised concentration of potentially neurotoxic A β dimers (Larson & Lesne, 2012). Therefore, we asked if neuronal RAR α signalling could help clear A β oligomers. Cortical neurons were cultured for 3 days with A β 42 oligomers (Fig. 5A), with or without AM 580. Immunostaining with 6E10 and RAR α showed that in A β 42 oligomer-treated cultures, abundant oligomers of A β surrounded the neuronal surface, whereas in the presence of AM 580 significantly fewer oligomers were seen (Fig. 5B and C, Student's *t* test, $t_6 = 8.311$, $P < 0.001$). This suggests that AM 580 induces A β clearance mechanisms in neurons. To confirm this, we first immunostained for NEP and IDE in the cultured neurons. This showed a cytoplasmic/perinuclear distribution in A β -treated cultures (Fig. 5D and F) but the enzymes became membrane-distributed upon treatment with AM 580, as in the vehicle-treated neurons. The major neuronal site for the degrading activity of both these enzymes

is the cell surface (Vekrellis *et al.*, 2000; Leissring *et al.*, 2003). We assayed for NEP activity as before, and for IDE activity, using neuronal membrane lysates. AM 580 significantly increased both enzymes' activity, reversing the A β -induced suppression (Fig. 5E and G, for NEP, one-way ANOVA followed by Fisher's test, $F_{3,8} = 7.835$, $P = 0.001$; for IDE, one-way ANOVA followed by Fisher's test, $F_{3,8} = 9.3$, $P = 0.002$).

RAR α signalling inhibits tau phosphorylation

A second hallmark of AD pathology is the abnormal hyperphosphorylation and intracellular accumulation of the microtubule-associated protein, tau, into neurofibrillary tangles. It is still unresolved in what ways the phosphorylation of tau contributes to toxicity, but recent work suggests that soluble intracellular oligomers, particularly dimers, of A β lead to phosphorylation of tau (Jin *et al.*, 2011) freeing it from the microtubule network and in consequence impairing some forms of intracellular transport, including that of mitochondria and some cargo vesicles (Ittner *et al.*, 2009; Kopeikina *et al.*, 2011). Thus, it is an important element of AD therapeutics that tau phosphorylation is inhibited. We therefore investigated the effect of AM 580 on tau phosphorylation. APPSWE/tau(P301L) transgenic mice were treated with vehicle, AM 580 or lithium for 3 months and their homogenized cortices were analysed by Western blotting. In the presence of AM 580 or lithium, phosphorylation was reduced at the AT8 and s396 (PHF-1) epitopes, compared with the vehicle-treated mice (Fig. 6A–C, for AT8, Student's *t*-test all against vehicle, $t_9 = 3.274$, vehicle vs. AM 580, $P = 0.01$, vehicle vs. lithium, $P = 0.01$; for s396, Student's *t*-test all against vehicle, $t_9 = 3.041$, vehicle vs. AM 580, $P = 0.014$; vehicle vs. lithium, $P = 0.013$). This was associated with an increased expression of phosphorylated GSK3 β at the serine 9 residue

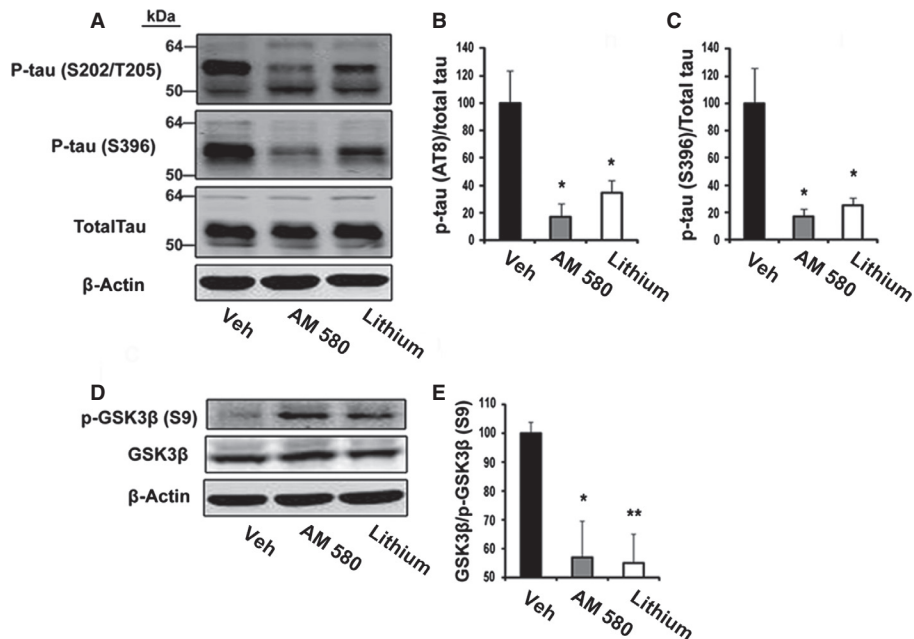


FIG. 6. AM 580 prevents tau phosphorylation in APPSWE/tau(P301L) mice. (A) Western blots of phosphorylated tau at epitopes Ser202/Thr205 (AT8) and Ser396 (S396) and total tau. (B) Quantitative analysis of protein levels shows a marked decrease in phosphorylation was observed at the AT8 (one-way ANOVA followed by Tukey's test, $*P < 0.01$). (C) S396 epitope of tau at 54 kDa in AM 580 and lithium compared with vehicle-treated mice. (D) Representative Western blots of phosphorylated GSK3 β at Ser9 [P-GSK3 β (Ser9)] and total GSK3 β . (E) Quantitative analysis of protein levels revealed increased levels of GSK3 β phosphorylated at the inhibitory epitope Ser9, as demonstrated by a decrease in the GSK3 β /P-GSK3 β (Ser9) ratio in AM 580 and lithium compared with vehicle-treated mice (one-way ANOVA followed by Tukey's test, $*P < 0.05$, $**P < 0.01$). Data are mean values expressed as a percentage of vehicle (set to 100%); $n = 3$ for all experiments. Error bars are SEM.

(Fig. 6D and E, Student's *t*-test all against vehicle, $t_{10} = 3.327$, vehicle vs. AM 580, $P = 0.008$; vehicle vs. lithium, $P = 0.002$).

Discussion

Retinoic acid receptor α is the predominant RAR expressed in the adult central nervous system (Corcoran *et al.*, 2004; Agudo *et al.*, 2010; Meng *et al.*, 2011) and it is becoming increasingly clear that it plays an important role in the maintenance of brain homeostasis (Lane & Bailey, 2005; Luo *et al.*, 2009; Duong & Rochette-Egly, 2011). We have shown here that RAR α signalling modulates key physiological mechanisms involved in A β clearance and how this signalling system is compromised by A β , which reduces the synthesis of the endogenous ligand RA. As RA and the RAR α system have been implicated as one mechanism contributing to homeostatic synaptic plasticity (Aoto *et al.*, 2008; Chen *et al.*, 2008; Maghsoodi *et al.*, 2008; Sarti *et al.*, 2012), the question should be raised as to whether A β also participates in this regulatory mechanism, given that normally synaptic activity sets its extracellular level (Cirrito *et al.*, 2005). There is evidence that endogenous A β has a number of physiological roles, including neuronal survival (Plant *et al.*, 2003), modifying the expression of potassium channels (Plant *et al.*, 2006) and of the probability of transmitter release (Abramov *et al.*, 2009) as well as being necessary for synaptic plasticity and memory (Puzzo *et al.*, 2008, 2011); most of these effects may be achieved by the monomeric form of A β .

The evidence for A β depression of RA production comes first from *in vivo* experiments showing this result for Tg2576 mouse brains. We do not know the concentration of A β reached in these brains, but administration of the RAR α agonist restores RA levels back to normal, wt, levels, compared with a ~50% reduction in the Tg2576 mice. We decided to explore microglial RA synthesis, as these cells are abundant in the AD brain (Hickman *et al.*, 2008) and play a role in the pathology of AD (Rogers *et al.*, 2002). In cultured microglia cells, RA synthesis was repressed in response to A β , cells became activated and significantly increased TNF α secretion. Co-treatment with an RAR α agonist reversed all these events and also correlated with an increase in apoE expression.

We have previously reported that in RA-deficient rats there is a reduction in the expression of RAR α (Corcoran *et al.*, 2004) and we show here that synthesis of RA is compromised in the cortices of Tg2576 mice. Thus, A β reduces the levels of both the agonist and the receptor and a good way to restore the level of RAR α is to administer an RAR α agonist. There is evidence that the same pathophysiology occurs in the AD brain, as we have reported that there is an approximately 30% reduction in RAR α levels compared with age-matched normal brains (Corcoran *et al.*, 2004).

Recent work has shown that retinoic acid-X receptor (RXR) ligands may have potential therapeutic in AD (Cramer *et al.*, 2012). However, to date no endogenous ligand of the RXR have been identified. Whilst 9-*cis* RA can activate RXRs this is very difficult to detect in animal tissues; alternative ligands such as unsaturated fatty acids have been shown to bind but with very low affinity (Wolf, 2006). Thus, activating a pathway that is not deficient in AD may lead to effects unrelated to AD. Here we have shown that the RA signalling pathway can be directly linked to A β , accumulation of which compromises RAR α signalling, which in turn can be restored by a specific RAR α agonist.

We have shown here that the RAR α signalling system upregulates several A β clearance mechanisms, in both neurons and microglia, including the important NEP proteolytic pathway, which is the only known mechanism for cleaving oligomeric amyloid (Nalivaeva *et al.*,

2012). We have also provided evidence that this retinoid system counteracts other potentially toxic effects of A β , such as impairment of cognitive behaviour, the release of a pro-inflammatory cytokine (TNF α) from microglia and the phosphorylation of tau. These observations should be put together with our earlier demonstration that activation of the RAR α pathway reduces the amyloidogenic production of A β and also acts neuroprotectively, reducing the A β -induced neuronal cell death in cortical cultures (Jarvis *et al.*, 2010).

We have shown previously that RAR α signalling will increase the activity of the non-amyloidogenic pathway and hence shed sAPP α into the extracellular medium. Amongst the actions of this molecule is amelioration of the calcium dyshomeostasis produced by A β (Mattson *et al.*, 1993) and the phosphorylation of GSK-3 β at the serine 9 site (Jimenez *et al.*, 2011). On the other hand, given the large number of neuronal genes that are potentially regulated by the retinoids (Lane & Bailey, 2005), it seems likely that other RAR α pathways are activated. This possibility then prompts the question about how extensive is the total list of effects produced by activating the RAR α pathway and, in particular, whether they would all be beneficial in the treatment of AD. Given that our evidence suggests that all the effects of the RAR α system will be equally down-regulated by A β in AD because it inhibits the synthesis of the endogenous agonist, RA, it would therefore be expected that all the effects would be a return towards normality.

An ideal therapeutic intervention would be to use an RAR α agonist at a dose which restores the RAR α to its normal level, giving this homeostatic mechanism in the brain an opportunity to reduce the production, enhance the clearance and counteract the untoward effects of excess A β , including the inhibition of the local synthesis of the endogenous ligand, RA. We regard the development of safe RAR α agonists, therefore, as a promising approach to arresting the pathophysiology of AD.

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Abbreviations

A β , amyloid beta; AD, Alzheimer's disease; apoE, apolipoprotein E; APP, amyloid precursor protein; DMSO, dimethyl sulfoxide; DSF, disulfiram; HFIP, 1,1,1,3,3,3 hexafluoro-2-propanol; IDE, insulin degrading enzyme; i.p., intraperitoneal; NEP, neprilysin; RA, retinoic acid; TNF, tumour necrosis factor; wt, wild-type.

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