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ORIGINAL ARTICLE

Activation of *Wnt* signaling by lithium and rosiglitazone reduced spatial memory impairment and neurodegeneration in brains of an APPswe/PSEN1ΔE9 mouse model of Alzheimer's disease

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive deterioration of cognitive abilities, accumulation of the amyloid-β-peptide (Aβ) and synaptic alterations. Treatment with lithium has been shown to provide neuroprotection against several insults, including protection against Aβ neurotoxicity *in vitro*. Rosiglitazone, a peroxisome proliferator activated receptor-γ agonist, has been shown to attenuate Aβ-peptide neurotoxic effects, including the inflammatory response of microglia and astrocytes. Both types of drugs activate *Wnt* signaling, a pathway that has been shown to be related to AD. In this study, a double transgenic mouse model, which coexpresses APPswe and the exon 9 deletion of the presenilin 1 (PSEN1) gene, was used to examine, *in vivo*, the effect of lithium and rosiglitazone on Aβ neurotoxicity. Mice were tested for spatial memory, and their brain samples were used for histochemical and biochemical analysis. In this study, we report that both drugs significantly reduced (1) spatial memory impairment induced by amyloid burden; (2) Aβ aggregates and Aβ oligomers; and (3) astrocytic and microglia activation. They also prevented changes in presynaptic and postsynaptic marker proteins. Finally, both drugs activate *Wnt* signaling shown by the increase in β-catenin and by the inhibition of the glycogen synthase kinase-3β. We conclude that lithium and rosiglitazone, possibly by the activation of the *Wnt* signaling pathway, reduce various AD neuropathological markers and may be considered as potential therapeutic agents against the disease.
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Introduction

Alzheimer's disease (AD) is characterized by a progressive deterioration of cognitive abilities, eventually leading to the individual's death.[1] Accumulation of the amyloid-β-peptide (Aβ), a product obtained by processing the amyloid precursor protein (APP), is believed to have a key function in the cognitive deficits observed in AD.[2] AD is also characterized as a synaptic disease that causes decreases in synaptic proteins and alterations in synaptic function.[3–6]

Lithium compounds are used in the treatment of various bipolar spectrum disorders, and have been shown to have neuroprotective effects against various insults.[7,8] In addition, they inhibit enzyme glycogen synthase kinase-3β (GSK-3β), leading to an increase in intracellular β-catenin and to the consequent activation of the *Wnt*–β-catenin signaling pathway.[9] Interestingly, the inhibition of GSK-3β has been shown to protect against Aβ neurotoxicity in mice models of AD.[10,11] Previously, we have shown in a rat model of AD, by directly injecting Aβ fibrils into the hippocampal regions, that lithium treatment resulted in the improvement of spatial memory and in decreased glial inflammatory reaction, most likely by the activation of the *Wnt* signaling pathway.[12] Moreover, therapeutic concentrations of lithium interfere with the APP cleavage at the γ-secretase level and reduce the amyloid burden in the brain of transgenic mice models of AD.[13,14]

The peroxisome proliferator activated receptor-γ (PPARγ) is a member of the family of PPAR nuclear receptors, with an important role in the regulation of lipid metabolism.[15] PPARγ agonists have been shown to attenuate Aβ neurotoxic effects by increasing the clearance mechanism of Aβ peptide or by cross talking with the *Wnt* signaling pathway.[16,17] Exposure of PC12 cells to ApoE4 (apolipoprotein E4) resulted in an inhibition of *Wnt* signaling,[18] and could therefore also reduce PPARγ activation. Moreover, it has been

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shown that treatments with rosiglitazone, a PPARγ agonist, in AD patients (ApoE4$^{-/-}$) lead to an improvement in cognitive test scores.[19] In addition, rosiglitazone improves learning and memory in transgenic mice with the Swedish mutation (APP K595N/M596L, *APPswe*).[20] Interestingly, rosiglitazone increases dendritic spine density and prevents spine loss caused by ApoE4 in cortical neurons,[21] indicating that it may have protective effects against the cognitive damage observed in AD.

In this study, we have examined the *in vivo* effects of lithium and rosiglitazone on Aβ neurotoxicity using a double transgenic mouse model that coexpresses APPswe and the exon 9 deletion of the presenilin 1 (*PSEN1*) gene.[22] Mice were tested for spatial memory, and their brain samples were used for biochemical and histochemical analysis. In this study, we report that lithium- and rosiglitazone-treated transgenic mice have significant improvements in spatial memory, and they showed lower levels of Aβ in hippocampal and cortical brain areas of both Aβ aggregates and Aβ oligomers. A reduced astrogliosis and microglial activation was also observed in both groups. In addition, the levels of presynaptic and postsynaptic marker proteins, which diminished in transgenic control mice, were maintained even after treatment with lithium and rosiglitazone.

Materials and methods

Reagents and antibodies

The following primary antibodies were used: rabbit anti-glial fibrillary acidic protein (GFAP) (DAKO, Carpinteria, CA, USA), mouse anti-Aβ (6E10, Chemicon, Temecula, CA, USA) and mouse monoclonal anti-postsynaptic density-95 (PSD95) (clone K28/43, obtained from Antibodies Inc.; Neuromab, Davis, CA, USA), rabbit anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-catenin (Santa Cruz Biotechnology), rabbit anti-VAMP1-2 (Santa Cruz Biotechnology) and rabbit anti-β-tubulin (Santa Cruz Biotechnology), rabbit anti-GSK-3β and anti-phospho-Ser9-GSK-3β (Cell Signaling Technology, Danvers, MA, USA). The reagents used were as follows: Rosiglitazone maleate (Avandia; GlaxoSmithKline, Brentford, UK) and lithium chloride (Sigma Chemicals, St Louis, MO, USA).

Subjects

Four-month-old *APPswe/PSEN1ΔE9* (known as APPPS1 henceforth in this study) bigenic mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA; Stock no. 004462). Wild-type (C57/BL) animals were also obtained from a colony of the Animal House Facility of the Facultad de Ciencias Biologicas (P. Universidad Catolica de Chile). All the animals were housed in temperature- and light-controlled rooms, with food and water *ad libitum* until the end of treatments. The animals were administered intraperitoneal (IP) injections of lithium chloride daily, dissolved in saline serum, or with rosiglitazone by oral gavage. Rosiglitazone maleate (Avandia) was ground and used as a suspension in sterile water. Control treatments included IP injections of saline solution or maleate in sterile water by gavage (0.45 mg ml^{-1}). There was no associated morbidity/mortality with daily IP injections of lithium at this dosage. No differences were found between transgenic mice and controls with IP injection, gavage or without treatment. The data of the transgenic control animals were polled and labeled as transgenic controls.

Behavioral test

The Morris water maze was performed as previously described in our laboratory.[12,23,24] The memory flexibility test was conducted as described by Chen *et al.*[25] Briefly, mice were trained in a circular water maze of 1.2 m diameter (opaque water, 50 cm deep, 19–21 °C, 9-cm platform 1 cm below water, maximum trial duration 60 s, 10 s on platform at the end of trials). Each animal was trained for one pseudo-random location of the platform per day, for 4 days, with a new platform location each day. Training was conducted up to 10 trials per day, until the criterion of 3 successive trials with an escape latency of <20 s was met. On completion of testing, the mouse was removed from the maze, dried and returned to its cage. The animals were tested for the next location on the following day. Data were collected using a video tracking system for water maze (HVS Imagen, Hampton, UK).

Immunohistochemical procedures

Perfusion and fixation were performed as previously described.[12,26] Free-floating immunohistochemical procedures were carried out as previously described.[26,27] Washing and dilution of immunoreagents were carried out using 0.01 M PBS (phosphate-buffered saline) with 0.2% Triton X-100 (PBS-T) throughout the experiments, with two PBS-T washes per antibody incubation. Sections were pretreated with 0.5% H_2O_2 for 30 min to reduce endogenous peroxidase activity, followed by treatment with 3% bovine serum albumin at room temperature for 1 h to avoid nonspecific binding. Primary antibodies were incubated overnight at 4 °C. They were detected using the Pierce ABC Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Staining was performed by incubating for 15 min with 0.6% diaminobenzidine, followed by the addition of H_2O_2 (0.01% final concentration). After immunostaining, all sections were mounted on gelatin-coated slides, air-dried, dehydrated and coverslipped with Canada balsam (Merck, Darmstadt, Germany). ThS (thioflavin S) staining was performed as previously described.[24,26,27] BSB ([(trans,trans)—1—Bromo—2,5—bis—(3—hydroxycarbonyl—4—hydroxy)-styrylbenzene) staining was performed as described earlier.[28,29] Briefly, the mounted sections were dehydrated and rehydrated in graded ethanol solutions, stained with BSB solution (200 μM) in 50% ethanol for 30 min, washed with

saturated solution of lithium carbonate, differentiated with 50% ethanol and covered using a fluorescent-mounting medium.

Immunoblotting

The hippocampus and cortex of treated or control transgenic mice were dissected on ice and immediately frozen in liquid nitrogen or processed as detailed previously.[30] Briefly, hippocampal and cortex tissues were homogenized in RIPA (radioimmunoprecipitation assay) buffer (10 mM Tris-Cl, pH 7.4, EDTA (ethylenediaminetetraacetic acid) 5 mM, 1% NP-40 (Nonidet P40), 1% sodium deoxycholate and 1% SDS), supplemented with a protease inhibitor mixture (1 mM PMSF (phenylmethanesulfonyl fluoride), 2 $\mu g\,ml^{-1}$ aprotinin, 1 $\mu g\,ml^{-1}$ pepstatin and 10 $\mu g\,ml^{-1}$ benzamidine) and phosphatase inhibitors (25 mM NaF, 100 mM Na_3VO_4, 1 mM EDTA and 30 μM $Na_4P_20_7$) using a Potter homogenizator, and then passed sequentially through different caliber syringes. Protein samples were centrifuged at 14 000 r.p.m. at 4 °C twice for 5 min. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). A total of 30 μg of hippocampal and cortex tissue samples were resolved by 12% SDS-PAGE, followed by immunoblotting on polyvinylidene difluoride membranes using mouse anti-PSD95 (Neuromab, Davis/NIH NeuroMab Facility, Davis, CA), rabbit anti-vesicle-associated membrane protein (VAMP), mouse anti-β-catenin, goat anti-synaptophysin, mouse anti-dishevelled-3 or rabbit anti-β-tubulin antibodies (Santa Cruz Biotechnology), rabbit anti-GSK-3β, rabbit anti-Ser9-GSK-3β (Cell Signaling Technology). Western blot assays were carried out as previously described.[31–33] Slot-blots assays were performed as previously described.[34] Briefly, the total protein extract was centrifuged to eliminate fibrillar aggregates at 20 000 g for 1 h. The protein concentration of the soluble fraction was determined and 6 μg of protein was spotted in 0.45 μm^2 nitrocellulose (Millipore, Bedford, MA, USA), blocked with PBS-T gelatin 0.4% and incubated using an anti-oligomeric antibody A11 (Chemicon) 1/5000 for 4 h at 4 °C. Slot blots were revealed with the same methodology as used for western blot.

Image analysis

The stained brain sections were photographed using an Olympus BX51 microscope coupled to a Micropublisher 3.3 RTV camera (QImaging, Surrey, BC, Canada). The luminance of the incident light and the time of exposure were calibrated to assign pixel values ranging from 0 to 255 in an red–green–blue image (no-light to full-light transmission), a setting that was used along all preparations. The images were loaded into ImageJ v.1.42d software (National Institutes of Health, Bethesda, MD, USA) for analysis. Selection of areas for measurement was performed by manual threshold adjustment. Quantifications were performed blindly in six coronal sections per animal, spaced 180 μm apart. Average optical densities were measured in 8-bit converted images. The analysis of each slot-blot picture was carried out by normalizing for the average intensity of control APP-PS1 slots in each experiment.

Statistical analysis

Data analysis was carried out using Prism software (GraphPad Software Inc., La Jolla, CA, USA). Results were expressed as mean ± s.e. For statistical analysis, normally distributed data were analyzed by one-way ANOVA (analysis of variance) with *post hoc* tests performed using the Tukey test. Non-normally distributed data were analyzed by the Kruskal–Wallis test, with *post hoc* tests conducted using Dunn's test.

Results

Treatments of lithium and rosiglitazone reduce spatial memory loss in double transgenic mice

Double transgenic mice that express mutant APP_{SWE} (K595N/M596L) and PS1 (PSEN1ΔE9: deletion of the exon 9) were used, producing depositions of Aβ plaques in the cortex and hippocampus in an age- and region-dependent manner.[35] These mice and age-matched wild types were used to analyze how physiopathological markers of AD were modified by lithium (3 $mequiv.\,kg^{-1}$ IP, daily) and rosiglitazone (3 $mg\,kg^{-1}$ gavage, daily) treatments initiated at 9 months of age. To determine the steady-state concentration of lithium in our regime of treatment, plasma lithium concentrations were measured in a separate group of animals. The therapeutic plasmatic concentrations are in the range of 0.2–1.5 $mequiv.\,l^{-1}$.[36] Plasma concentration was 0.225 $mequiv.\,l^{-1}$ 24 h after lithium injection (Supplementary Information, Figure 1), below toxic concentrations and in the range of therapeutic concentration.[37]

Hippocampal function was tested through a spatial memory test known as the Morris water maze. After 12 weeks of treatment, high variability was observed, and no clear difference among the animals groups was detected (Figure 1a). Therefore, a more sensitive version of the Morris water maze, which measures memory flexibility, was evaluated[25] This modified version of the spatial memory Morris water maze has proven to be more sensitive to hippocampal dysfunctions.[25,38] In this task, mice are required to learn a series of spatial locations successively, one at the time, testing for an episodic-like component of encoded location.[39] A deficit in the number of trials required to reach the escape criterion was found at each of the four platform locations for the APP-PS1 control mice. As observed in Figure 1b, treated animals required a significantly reduced number of trials to reach criterion, and this improvement in spatial memory almost reached wild-type levels (Figure 1c). A *post hoc* analysis of the data shows that treated APP-PS1 animals have improved memory flexibility than untreated APP-PS1 mice. When the strategies used by the animals to reach the platform were compared at day 4 (times < 20 s), it was possible

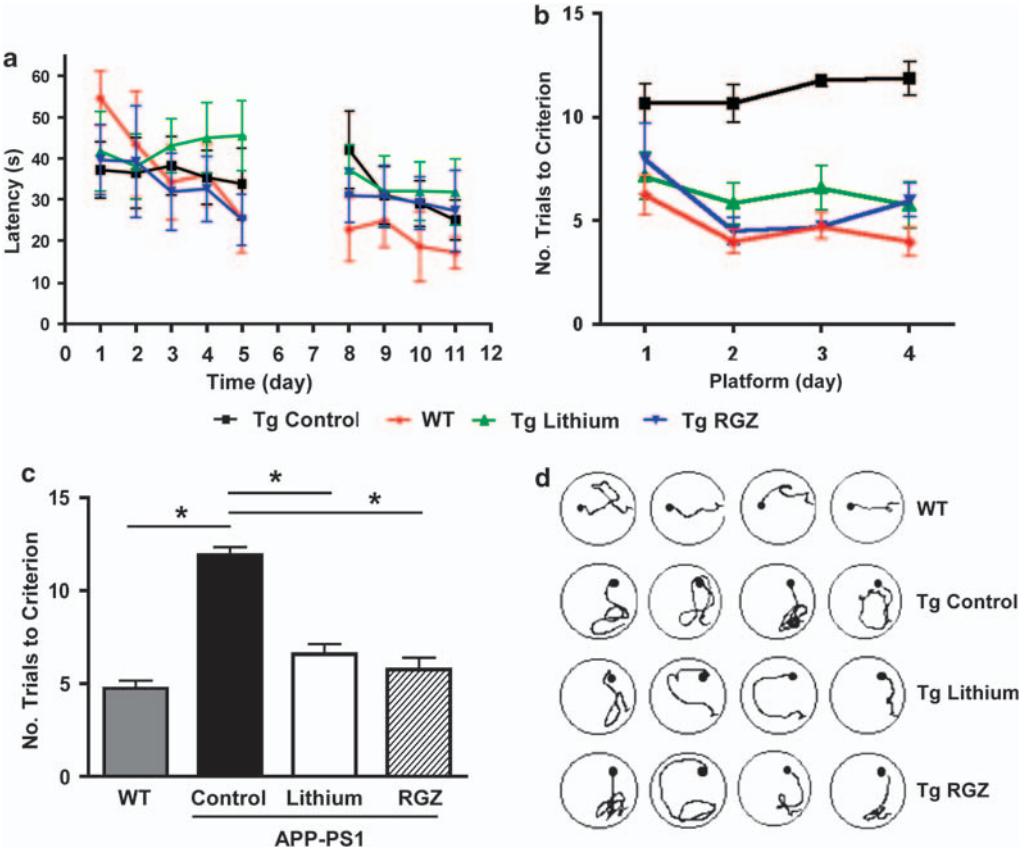

Figure 1 Treatments with lithium and rosiglitazone improve memory flexibility. Amyloid precursor protein (APP)-PS1 animals were treated with lithium and rosiglitazone for 12 weeks. (**a**) Highly variable results were obtained in classic Morris water maze. (**b**) Daily progression of the memory flexibility test on each platform location (**c**) An increase in the number of trials necessary to reach the criterion is observed in APP-PS1 animals versus wild-type animals. Treatment with lithium (daily doses of 3 mequiv. kg^{-1}) and rosiglitazone (daily doses of 3 mg kg^{-1}) reduces the number of trials necessary to reach the criterion. (**d**) Final tracks of the last day of testing show different swimming strategies of the animals. Asterisks indicate significant difference, $^{*}P<0.05$.

to appreciate the difference between wild-type animals and APP-PS1 controls. In fact, treated animals showed improved strategies compared with APP-PS1 controls. Treated APP-PS1 animals presented strategies that were more similar to those by wild-type animals than the transgenic controls (Figure 1d). Our behavioral analysis indicates that lithium and rosiglitazone reduce the deficit in spatial memory observed in these APP-PS1 transgenic mice.

Aβ plaque size in APP-PS1 mice is reduced with lithium and rosiglitazone treatment

To examine whether the improvement in mice behavior was correlated with a change in AD neuropathological markers, we analyzed the amount of Aβ plaques present in the brains of APP-PS1 animals. Brain sections obtained from lithium-, rosiglitazone-and vehicle-treated mice were stained with an antibody against Aβ and analyzed for Aβ burden present in cortical and hippocampal regions (Figure 2a–f). The APP-PS1 brain sections showed a reduction in the positive area for Aβ aggregates in treated animals versus controls in the cortex and hippocampus (Figure 2g and h). A detailed analysis of the plaque size distribution of the Aβ area was also carried out. The aggregate size distribution was presented as a cumulative plot, which showed that APP-PS1 mice treated with lithium and rosiglitazone had an increased number of small plaques in both the cortex ($0.6<mm^2$) and hippocampus, although still maintaining smaller overall plaque loads in these regions (Figure 2i–j). With the use of antibodies against Aβ, all kinds of positive areas in the cortex and hippocampi were detected and considered in the quantification.

To identify whether lithium or rosiglitazone alters the amount of amyloid β-sheet deposits present, brain sections from wild-type mice, APP-PS1 control, APP-PS1 lithium- and APP-PS1 rosiglitazone-treated mice were stained with ThS and analyzed for the area fraction positive to ThS and plaque density (Figure 3a–f). This analysis showed a reduction in the positive area to ThS and in the number of plaques per area in both treatments (Figure 3g and h). This result is in agreement with the findings that lithium- and rosiglitazone-treated groups had a reduction in the overall amount of Aβ aggregates detected using antibodies against Aβ.

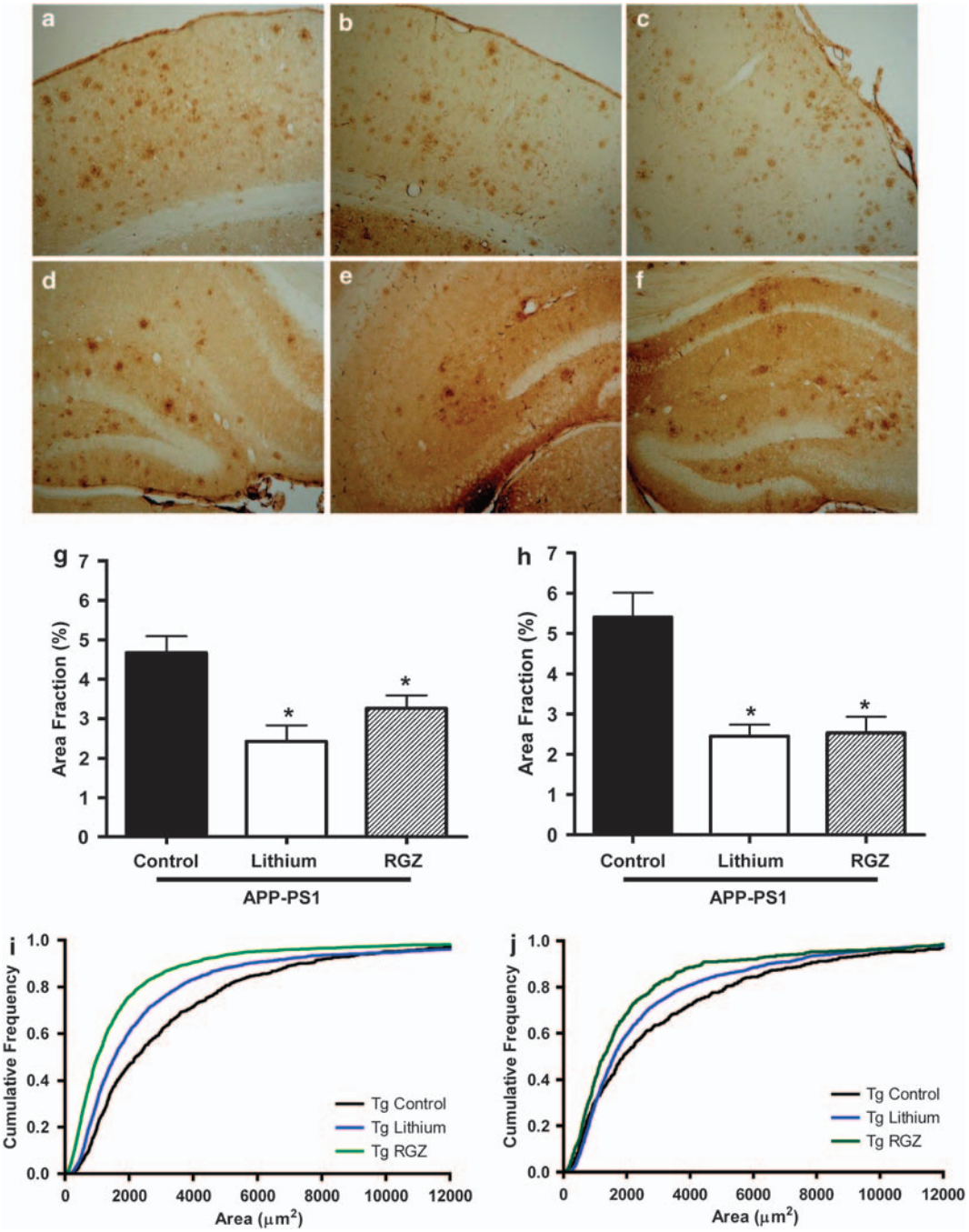

Figure 2 Lithium and rosiglitazone reduce the amount of total Aβ in brains of transgenic (Tg) mice. Cortical (**a–c**) and hippocampal (**d–f**) slices obtained from amyloid precursor protein (APP-PS1) mice stained against amyloid-β-peptide (Aβ), using antibody 6E10. Pictures are of APP-PS1 controls (panels a and d), APP-PS1 treated with lithium (panels b and e) and APP-PS1 treated with rosiglitazone (panels c and f). (**g** and **h**) The average area fraction positive to Aβ in the cortex and hippocampus, respectively is shown, with bars representing the average plaque area for each specific treatment and error bars representing ± s.e. ($n \geqslant 6$). Asterisks indicate significant difference, $^*P < 0.05$. Each histogram (**j**, panel h) corresponds to the figure above it and represents the different frequencies of Aβ plaque sizes (μm²) for APP-PS1 control, lithium and rosiglitazone treatments, respectively. Asterisks indicate significant difference, $^*P < 0.05$.

Lithium and rosiglitazone treatment reduces inflammatory reaction in double transgenic mice

In an attempt to understand the mechanism by which lithium and rosiglitazone affect Aβ aggregates, the astroglial inflammatory reaction normally observed in brains with Aβ plaques was studied. Brains sections from vehicle-, lithium- and rosiglitazone-treated mice were stained against GFAP and analyzed for the presence of hypertrophic glial cells (Figure 4a–h). A detailed analysis was conducted including the

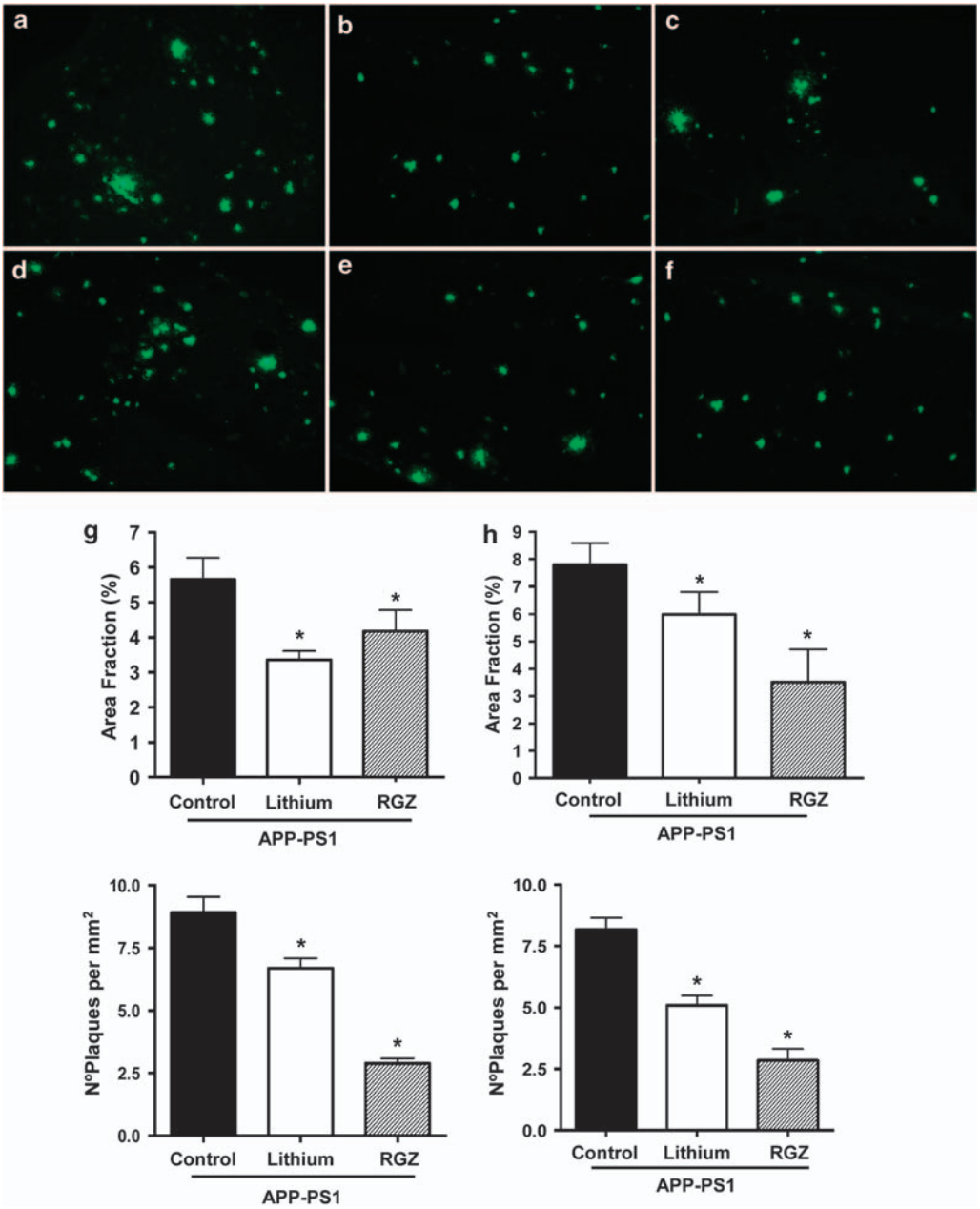

Figure 3 Staining of ThS (thioflavin S) in amyloid-β-peptide (APP-PS1) mice brains. Cortical (**a–c**) and hippocampal (**d–f**) slices obtained from APP-PS1 mice stained with ThS. Pictures are of APP-PS1 control (panels a, d), APP-PS1 mice treated with lithium (panels b, e), APP-PS1 mice treated with rosiglitazone (panels c, f). The average area fraction (upper panels) positive to ThS and the number of plaques per area (lower panels) in the cortex (**g**) and hippocampus (**h**), with bars representing the average for each specific treatment and error bars representing ± s.e. ($n \geqslant 6$). Asterisks indicate significant difference, $^*P < 0.05$.

average perikaryon area and the average GFAP intensity in the measured area. The perikaryon area of astrocyte cells was significantly decreased in size in mice treated with lithium and rosiglitazone in cortical (Figure 4i, upper panel) and hippocampal regions (Figure 4j, upper panel), in comparison with that in APP-PS1 control mice ($63.63 \pm 2.03\,\mu m^2$; $71.34 \pm 1.68\,\mu m^2$), reducing them to levels similar to those of wild-type mice ($39.91 \pm 1.71\,\mu m^2$; $40.63 \pm 0.91\,\mu m^2$). Astroglial GFAP intensity in the cortex and hippocampus was also significantly reduced in lithium- and rosiglitazone-treated mice in comparison with that in control transgenic mice, and showed intensities similar to those of wild-type mice (Figure 4i and j lower panels). The microglial component of the inflammatory response was also analyzed. Sections of all experimental groups were stained against the CD11b marker. The analysis of the

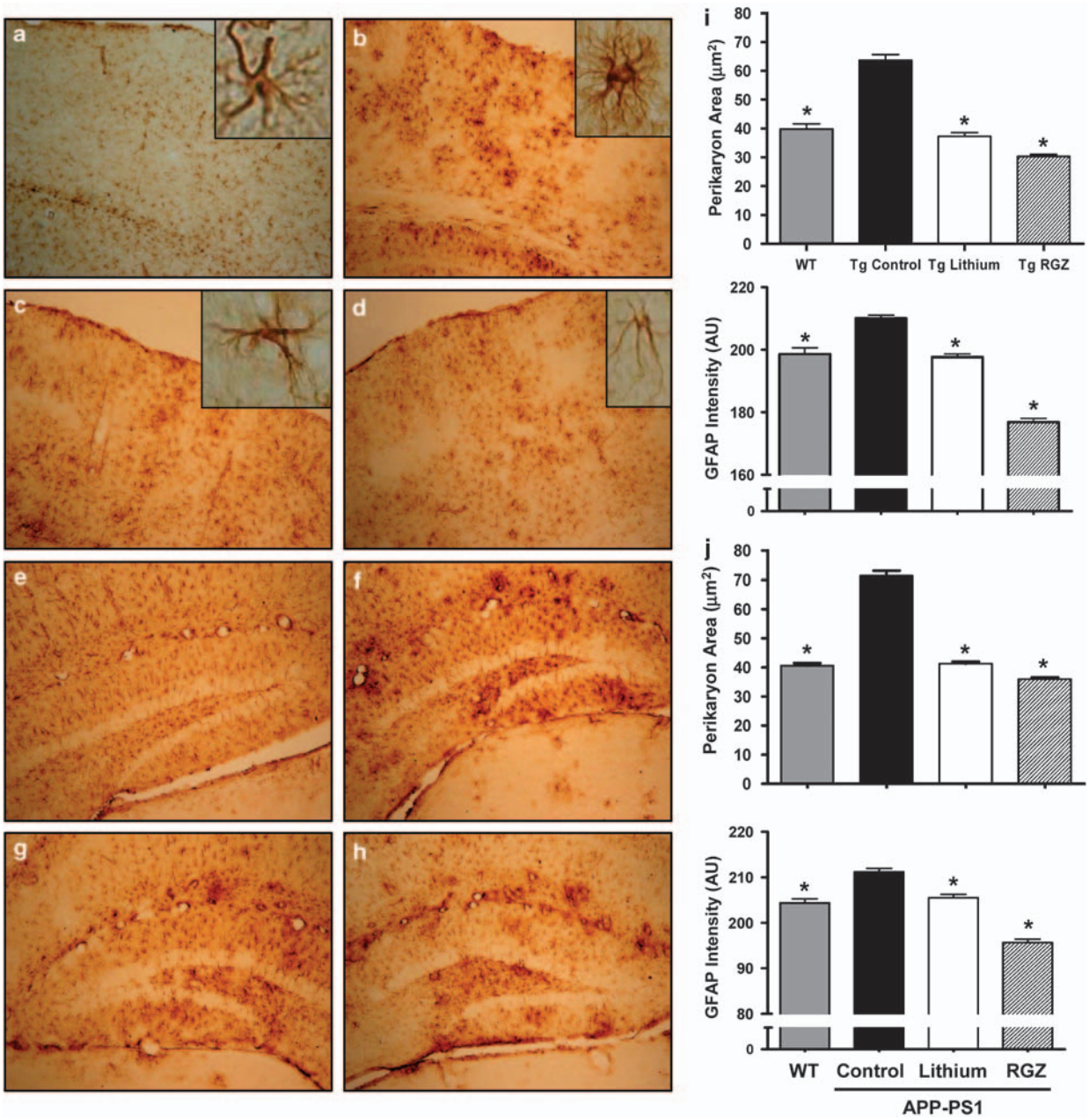

Figure 4 Lithium and rosiglitazone reduce astrogliosis in brains of amyloid-β-peptide (APP)-PS1 mice. Histochemistry against GFAP in cortical (**a–d**) and hippocampal (**e–h**) slices from wild-type and APP-PS1 mice. Photos are of wild-type controls (panels a and e), APP-PS1 control mice (panels b and f), APP-PS1 treated with lithium (panels c, g) and APP-PS1 treated with rosiglitazone (panels d, g). (**i** and **j**) The average perykarion area of glial cells (upper panel) and the GFAP intensity in the measure perikaryons is shown (lower panels) in the cortex (panel i) and hippocampus (panel j), with bars representing the average for each specific treatment and error bars representing ± s.e. ($n \geqslant 6$) Asterisks indicate significant difference, $^{*}P < 0.05$.

area that is positive for CD11b showed that the area fraction of control APP-PS1 mice was significantly higher in comparison with that in treated animals (Figure 5a and f), in the cortex (Figure 5g) and hippocampus (Figure5h). The above results indicate that the inflammatory response found in APP-PS1 mice is reduced on treatment with lithium and rosiglitazone.

Lithium and rosiglitazone recover the loss of synaptic proteins

Previous studies have indicated that synaptic disturbances are present in AD brains and in transgenic animal models.[3–6,40] To further analyze the implication of lithium and rosiglitazone in the neuroprotection observed in our study, we analyzed the amount of various presynaptic and postsynaptic proteins de-

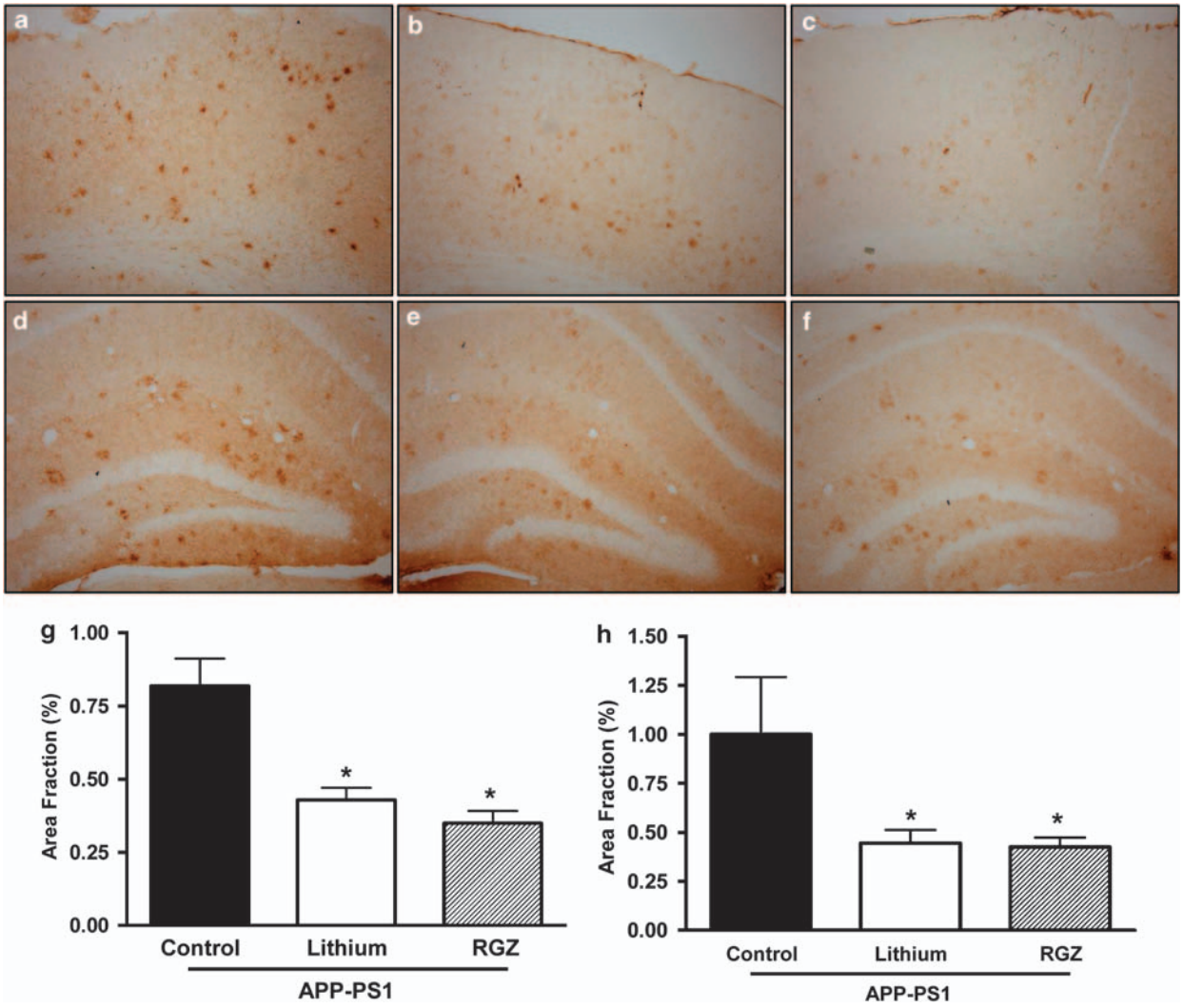

Figure 5 Lithium and rosiglitazone reduce activated microglia in brains of amyloid-β-peptide (APP)-PS1 mice. Histochemistry against the microglial marker CD11b in the cortex (**a, c**) and hippocampus (**d, f**) of APP-PS1 mice control (panels a, and d), APP-PS1 mice treated with lithium (**b, e**) and APP-PS1 treated with rosiglitazone (panels c and f). The analysis of the stain shows that both treatments reduce the amount of activated microglia in the cortex (**g**) and hippocampus (**h**). ($n \geqslant 6$). Asterisks indicate significant difference, $^{*}P < 0.05$.

tected in the hippocampus of APP_{swe}/PSEN1ΔE9 mice. The presynaptic proteins, synaptophysin and VAMP, as well as the postsynaptic protein, PSD-95, showed an increase in total levels after lithium and rosiglitazone treatments (Figure 6), as can be seen in the densitometry analysis.

Considering the fact that the target for Aβ oligomers is the postsynaptic region,[41,42] the distribution of PSD-95 in the hippocampus was analyzed using histochemistry. High levels of PSD-95 are present in the hippocampal region of wild-type animals and, when compared with APP-PS1 control animals, it is possible to detect a loss of PSD-95 staining in the CA3 stratum lucidum region of the hippocampus (Figure 7a and b). However, this change was recovered in brains sections of treated animals (Figure 7c and d), as can be observed in the densitometry analysis of these regions (Figure 7e), thus indicating that both treatments are capable of preventing the decrease in postsynaptic proteins found in the hippocampus of APP-PS1 animals.

Wnt *signaling activation reduces the amount of A*β *oligomers in the hippocampus*

As oligomeric aggregates of Aβ have proven to be synaptotoxic[4] and reduce synaptic function *in vitro* and *in vivo*,[42–45] we decided to evaluate the amount of Aβ oligomers present in brains of APP-PS1 mice. First, we assessed the areas and distribution of aggregate positives with BSB, a derivative of Congo Red that is more prone to stain oligomers than fibrils.[29] Brain sections from wild-type mice, APP-PS1 control, APP-PS1 lithium- and APP-PS1 rosiglitazone-treated mice were stained with BSB and analyzed for area fraction and plaque density (Figure 8a–f). This analysis showed a reduction in the

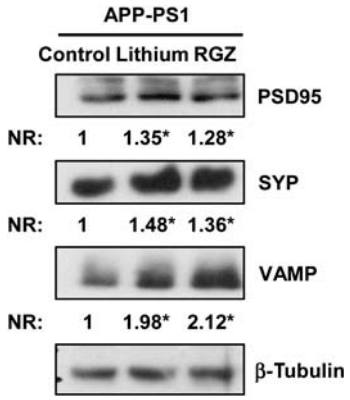

Figure 6 Lithium and rosiglitazone prevent the reduction of synaptic markers in the hippocampus of amyloid-β-peptide (APP)-PS1 mice. Representative gels of total protein from hippocampus show the protection of the reduction of the amount of synaptic proteins, PSD-95, synaptophysin and VAMP, from extracts of APP-PS1 mice, control and from mice treated with lithium and rosiglitazone. Normalized ratio (NR) of the densitometric analysis of each treatment normalized against the level of β-tubulin and compared against levels of APP-PS1 control mice($n \geqslant 3$). Asterisks indicate significant difference, $^*P < 0.05$.

positive area of BSB staining in the hippocampus by both treatments (Figure8h). However, in the cortex, we did not find a change in the area of positive aggregates with both treatments (Figure 8g). Neither treatment showed a reduction in the numbers of positive aggregates per area (data not shown). Slot-blot assays were carried out to assess the amounts of Aβ oligomers detected by the antibody A11, which detect oligomeric aggregates with a mass over 40 kDa.[46] Hippocampal extracts obtained from APP-PS1 animals, control and treated, were blotted in a nitrocellulose membrane and detected with the A11 antibody (Figure 8i). The densitometry analysis showed a significant reduction in the levels of oligomeric species detected in the hippocampi of APP-PS1 mice with both treatments (Figure 8j).

The reduction of Wnt *signaling is prevented by lithium and rosiglitazone*

Previous studies in our laboratory have indicated that *Wnt* signaling activation is related to neuroprotection against Aβ injury.[5,12,31,47–50] Considering that lithium is a pharmacological activator of the *Wnt* signaling pathway and rosiglitazone is a PPARγ agonist that activates *Wnt* signaling by cross talk,[5,17] we wanted to confirm that under our experimental conditions, *Wnt* signaling was in fact activated. The expression of *Wnt* key proteins was analyzed by western blotting. These results indicated that APP-PS1 mice have lower levels of Dvl-3 and β-catenin, and increased levels of activated GSK-3β than do wild-type animals (Figure 9). These results showed a reduction in canonical *Wnt* signaling in this model of AD. The APP-PS1 mice treated with lithium showed a recovery in the levels of β-catenin and Dvl-3, and increased levels of the inhibited GSK-3β (Figure 9). Rosiglitazone showed a clear recovery in the levels of β-catenin and increased levels of the inactive GSK-3β; however, no effect was observed in Dvl-3 levels. These results strongly suggest that lithium and rosiglitazone activate *Wnt* signaling *in vivo*.

Discussion

In this study, we showed that transgenic mice APPswe/PSEN1ΔE9 had a deficit in spatial memory, which could be partially restored to wild-type levels by treatment with both lithium and rosiglitazone. The observed increase in Aβ loads as well as the increased astrogliosis present in the transgenic control mice, is correlated with impairment in their spatial memory. However, treatment with lithium and rosiglitazone prevents the aforementioned changes.

Lithium is a common drug used for the treatment of bipolar spectrum disorders,[51] and it possesses a great potential for the treatment of numerous other pathologies.[52] One of lithium's interesting mechanisms of action is its inhibition of the enzyme GSK-3β, which mimics the effect of activation of canonical *Wnt* signaling.[9,53] Previously, it was found that treatment with lithium protects against different insults;[7,54] however, the relationship between an inhibition of this enzyme and lithium *in vivo* has been insufficiently explored. Lithium was previously reported to reduce the amount of phosphorylated τ *in vitro*[55] and *in vivo*.[10,56] Using a transgenic model of AD, it was shown that the decrease in the phosphorylation of τ occurs without affecting the amount of Aβ plaques or memory impairment.[57] However, those studies were carried out in a triple transgenic model that expresses a mutated form of the τ-protein, in addition to the mutated forms of APP and PS1. It is quite possible that overexpression of a mutated form of τ-protein is a pathological feature that the treatment of lithium cannot overcome.

Peroxisome proliferator activated receptor-γ agonists have been found to protect against Aβ toxicity in neurons.[17,58,59] Mechanisms that have been associated with some PPARγ agonists include decreased glial inflammation through the decreased expression of proinflammatory enzymes, such as cyclooxygenase 2 and the inducible nitric oxide synthase.[60] The underlying mechanism of rosiglitazone remains unclear. Although rosiglitazone is capable of increasing the clearance mechanism of the Aβ peptide,[16] activation of the *Wnt* signaling,[17] it is not clear whether the neuroprotection attributed to rosiglitazone is because of its PPARγ agonist activity or its downstream effects. At least three possible scenarios can be attributed to its neuroprotective effects: first, a direct activation of PPARγ genes; second, non-genomic effects of the PPARγ activation; and finally, cross talk with another pathway, that is, the *Wnt* pathway. Further research is necessary to analyze the presented possibilities. The APP-PS1 mice treated with rosiglitazone did not show increased levels of Dvl-3, but showed increased levels of β-catenin and the inhibited form of GSK-3β,

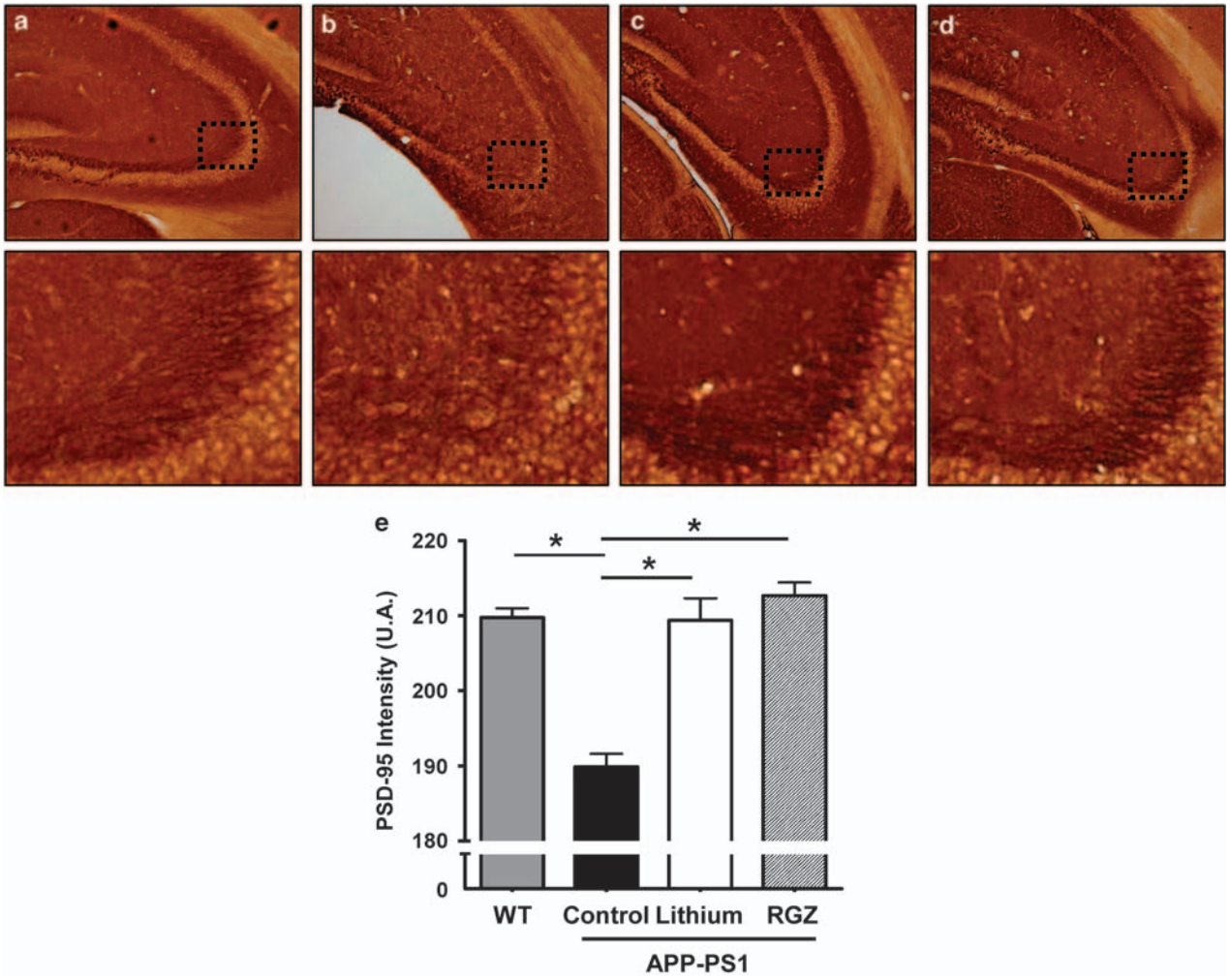

Figure 7 Lithium and rosiglitazone prevent the reduction of PSD-95 in the stratum lucidum of amyloid-β-peptide (APP)-PS1 mice. Histochemistry against PSD-95 in brain slides from wild type (**a**), APP-PS1 control (**b**), APP-PS1 treated with lithium (**c**) and APP-PS1 treated with rosiglitazone (**d**). Analysis of the staining shows that both treatments prevent the reduction of PSD-95 found in the CA3 stratum lucidum of the hippocampus. Bottom images, amplification of the dotted box. (**e**) Quantification of the average intensity of the staining of PSD-95 in the stratum lucidum ($n \geqslant 6$). Asterisks indicate significant difference, $^*P < 0.05$.

and as Dvl-3 is activated right after the binding of *Wnt* to the receptor,[61,62] this result may help to reveal a part of the mechanism of the cross talk.[63]

Our results indicate that APP-PS1 mice treated with lithium and rosiglitazone had a significantly lower area occupied by plaques than did transgenic control mice. Furthermore, when plaques were analyzed for their size distribution, we observed that APP-PS1 mice treated with lithium and rosiglitazone had a higher amount of small plaques ($< 0.5\,mm^2$) in both the cortex and hippocampus as compared with transgenic controls. It was previously reported that lithium has the ability to inhibit Aβ production, limiting plaque growth.[13] Furthermore, PPARγ agonists have shown their ability to reduce levels of secreted Aβ *in vitro*.[16] The potential mechanism to limit the size of Aβ found in both treatments could be attributed in part to a reduced production of the Aβ peptide.

The analysis of the nature of plaques present in transgenic brains indicated that treatments reduce the amount of total aggregates, plaques and oligomers, suggesting that the reduction observed is probably because of an increase in disassembly, clearance and/or catabolism of Aβ aggregates, and therefore it is possible that *Wnt* signaling activation increases an active Aβ clearance mechanism that, under normal conditions, could be found at a basal activity.[64]

Of the described mechanism of Aβ catabolism,[65] the observed effect could be attributed to metalloproteinases (MMPs). MMP activity, mainly in astrocytes and microglia, is able to degrade oligomeric and fibrillar aggregates.[65–67] Furthermore, MMP-2 and MMP-9 are identified as *Wnt* target genes in T cells;[68] MMP-7 is a *Wnt* target gene in colon rectal cancer.[69,70] Moreover, the inhibition of GSK-3β by Akt leads to an increased level in the activity of MMP.[71] All this evidence suggests that *Wnt* activation could restore a

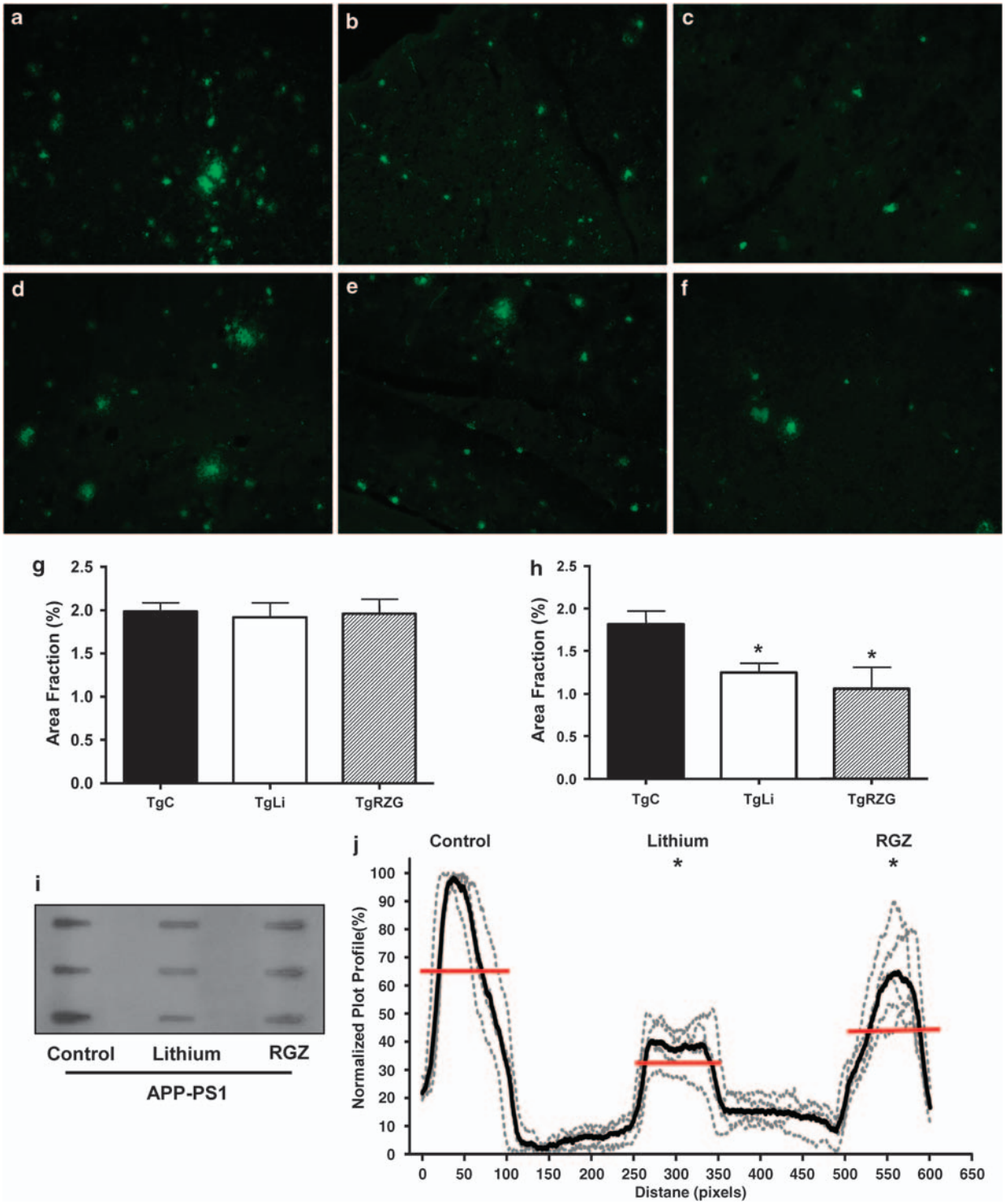

Figure 8 Determination of oligomers in amyloid-β-peptide (APP)-PS1 brains. Cortical (**a–c**) and hippocampal (**d–f**) slices from APP-PS1 mice stained with BSB. Pictures are of APP-PS1 control (panels a and d), APP-PS1 mice treated with lithium (panels b and e), APP-PS1 mice treated with rosiglitazone (panels c and f). The average area fraction positive to BSB in the cortex (**g**) and hippocampus (**h**), with bars representing the average for each specific treatment and error bars representing ± s.e. ($n \geqslant 6$). Slot-blot analysis from hippocampal extracts using anti-oligomeric antibody A11. Representative slot blot picture, 6-μg protein per slot was spotted to a nitrocellulose membrane and exposed to the A11 antibody (**i**). Normalized densitometric analysis profile of each slot intensity versus distance (dotted lines), average line (black bold line) and red line average intensity of each treatment (100 pixels wide) (**j**). Asterisks indicate significant difference, $^*P < 0.05$.

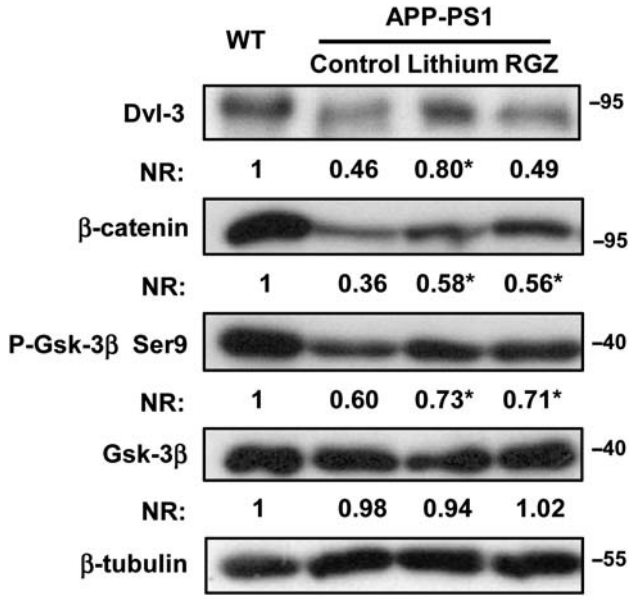

Figure 9 Lithium and rosiglitazone prevent the reduction *Wnt* signaling in the hippocampus of amyloid-β-peptide (APP)-PS1 mice. Representative gels of total protein from hippocampus show the recovery of the reduction of the amount of synaptic proteins Dvl-3, β-catenin, p-GSK-3β and total GSK-3β from extracts of wild-type and APP-PS1 mice, of control, and of mice treated with lithium and rosiglitazone. Densitometric analysis of each treatment normalized against the level of β-tubulin and compared against levels of APP-PS1 control ($n \geqslant 3$). Asterisks indicate significant difference, $^{*}P < 0.05$.

catabolic pathway found in glial cells, probably through the action of MMP but without ruling out the actions of other catabolic activities.

Increased gliosis is a characteristic feature of brains with increased Aβ loads.[72,73] In this study, we observed that APP-PS1 control mice had a significantly increased perykarion size and GFAP intensity, which was decreased to wild-type levels in APP-PS1 mice treated with lithium and rosiglitazone. This may indicate that lithium and rosiglitazone can protect against the glial inflammatory reaction observed in AD brains, which may amplify the damage already caused by Aβ plaques. In addition, the prevention of the decrease in synaptic markers found in treated mice showed that lithium and rosiglitazone prevent synaptic failure found in models of AD.[42,43,74,75] These changes correlate with an enhanced performance in our memory flexibility test, suggesting that hippocampal synaptic function is improved under both treatment conditions.

Increasing evidence has shown a relationship between *Wnt* signaling and synaptic functions. In fact, we found that *Wnt*/β-catenin modulates the synaptic vesicle cycle and synaptic transmission in rat hippocampal neurons,[76] and non-canonical *Wnt* signaling also modulates PSD-95 clustering.[33] Therefore, these possible mechanisms needed to be explored in APP-PS1 animals treated with lithium and rosiglitazone. Recently, He and Shen[77] reported that the renewal capacity of glial progenitor cells isolated from AD patients is reduced compared with that of cells from healthy controls, and this reduced neurogenesis capacity was correlated with GSK-3β activity and an increased phosphorylation of β-catenin. They also found that treating glial precursor cells from healthy controls with Aβ also led to increased β-catenin phosphorylation and reduced neurogenesis. This finding suggests that, in AD patients, Aβ impairs neurogenesis and *Wnt* signaling has a key function in these effects.

The one question that we were unable to address in this study is whether one or both treatments can overcome the neurotoxicity of Aβ at the level of synaptic spines. Dendritic spines have been found to be targets of oligomeric Aβ,[6,43] and *in vivo* APP and PS1 transgenic mice develop a reduction in spine density because of Aβ.[45] The analysis that rosiglitazone treatments protect against the toxicity of an ApoE4 fragment and increase dendritic spine density[21] suggests that this could be a mechanism by which rosiglitazone overcomes synaptic impairment.

In summary, this study indicates that lithium and rosiglitazone treatment improves the disturbances observed in memory flexibility in the APP-PS1 model of AD. These results correlated with a reduction in the amount of Aβ aggregates present and a concomitant decrease in glial inflammatory reaction, as well as an increase in presynaptic and postsynaptic proteins. More interestingly, both these compounds may induce an activation of the *Wnt* signaling pathways. Treated APP-PS1 mice with lithium showed increased levels of Dvl-3, and both treatments showed increased levels of β-catenin and the inhibited state of GSK-3β in APP-PS1 mice; hence, the aforementioned results could be an outcome of the increase in *Wnt* signaling. Our results show neuroprotection by *Wnt* signaling in an *in vivo* transgenic mouse model. Finally, our study shows that both lithium and rosiglitazone have the potential to uncover new therapeutic possibilities for the treatment of AD.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)