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Simvastatin Treatment Preserves Synaptic Plasticity in AβPPswe/ PS1dE9 Mice

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Abstract. Epidemiological evidence suggests that chronic treatment with simvastatin may protect against the development of Alzheimer's disease (AD), but as yet it is unclear how this effect is mediated. Extensive data also indicates that the amyloid β-protein (Aβ) plays a central role in the disease process, and it has been suggested that the protective effects of simvastatin may be mediated by reducing Aβ production or by counteracting the toxic effects of Aβ. Accordingly, using the AβPPswe/PS1dE9 mouse model of AD, we investigated the effects of simvastatin on long-term potentiation (LTP), amyloid biology, and two key kinases involved in Aβ-mediated toxicity. Since burgeoning data indicate that both fibrillar and non-fibrillar forms of Aβ play a prominent role in AD pathogenesis, we were careful to investigate the effects of simvastatin on three biochemically distinct pools of Aβ. In untreated AβPPswe/PS1dE9 mice, there was a dramatic and significant increase in the levels of water-soluble Aβ between 6 and 8 months, but this remained constant between 8 and 18 months. In contrast, the concentrations of detergent-soluble and formic acid (FA)-soluble Aβ species increased across all ages examined, thus demonstrating that while amyloid deposition continued, the levels of water-soluble Aβ remained relatively constant. LTP was normal at 6 months, but was significantly impaired at 8 and 18 months. Importantly, a diet supplemented with 0.04% simvastatin for one month (at 7 months) positively affected synaptic plasticity in AβPPswe/PS1dE9 mice and did not significantly alter levels of water-soluble, detergent-soluble, or FA-soluble Aβ, but did increase phosphorylation of both Akt and GSK-3, while tau and tau phosphorylation were unaltered. These results indicate that the protective effects of simvastatin may be mediated by maintaining signaling pathways that help to protect and rescue LTP.

Keywords: Alzheimer's disease, amyloid-β, hippocampus, long-term potentiation, statin

INTRODUCTION

Statins are HMG-CoA reductase inhibitors used to treat hypercholesterolemia, and it is known that some statins, including simvastatin (SV), can penetrate the blood-brain barrier [1, 2]. There have been numerous reports on the pleiotropic effects of statins. Studies report that SV has anti-inflammatory properties [3] and can also improve learning and memory performance in rodents [4, 5]. Several epidemiological investigations have also shown beneficial outcomes of statin treatment in stroke, dementia, and Parkinson's disease [6–10]. Controversy still exists as to the potential therapeutic benefit of statin treatment for Alzheimer's disease (AD). Positive effects have been reported by some groups [7, 8, 11, 12], while other studies have reported little if any effect [10, 13–15].

AD is the most common neurodegenerative disease in our aging population. Two characteristic hallmarks of AD are plaques of aggregated amyloid β-protein (Aβ) and neurofibrillary tangles formed from hyper-

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phosphorylated tau. The so-called "amyloid cascade hypothesis" [16, 17] appears to best explain what we know about AD pathogenesis and has dominated molecular research on the disease for the past two decades. The foundation of the this hypothesis rests on evidence that increased production or decreased clearance of Aβ leads to the generation of toxic assemblies which initiate a complex cascade of molecular events that culminate in frank dementia [18]. Aβ is a normal physiological product which is generated from a precursor protein, the amyloid-β protein precursor (AβPP) [19]. Extensive evidence indicates that Aβ production is strongly influenced by cholesterol [20], with cholesterol depletion reducing Aβ production [21] and increased intracellular cholesterol levels increasing Aβ production [22]. The precise molecular mechanism by which cholesterol mediates these effects are not yet fully understood, but it seems likely that this involves the modulation of proteases which process AβPP [23, 24], trafficking of AβPP [25–27], and regulation of Aβ degradation [28]. Cholesterol has been shown to bind C99, the transmembrane carboxyterminal domain of AβPP which may alter AβPP processing to promote amyloidogenisis [29]. Cholesterol may also influence the aggregation of Aβ [30], the process by which toxic assemblies of Aβ are formed.

Long-term potentiation (LTP) is a long-term activity dependent enhancement of synaptic strength that is believed to be involved in learning and memory processes and is exquisitely sensitive to toxic assemblies of Aβ [31]. Several signaling pathways have been implicated in the induction and in the maintenance of LTP. Akt has been linked to neuronal survival mechanisms and synaptic plasticity processes. Recently, Aβ has been shown to disrupt LTP through a signaling pathway that involves Akt1, GSK3β, and caspase-3 [32]. GSK3β is also known to be regulatory gating element for LTP and long-term depression (LTD) [33]. GSK3 expression is upregulated in the hippocampus of AD patients [34] and has been reported to co-localize with dystrophic neurites and neurofibrillary tangles [35–37]. We investigated the effect of chronic SV treatment (40 mg/kg/d) on LTP in a mouse model of AD using extracellular field potential recordings in the CA1 region of the hippocampus. Levels of Aβ were assessed by immunoprecipitation/quantitative western blotting. Western blot was also used to examine Akt and GSK3. Our results show that chronic SV treatment rescued the LTP deficits in 8 month old AβPPswe/PS1dE9 mice and that this involved increased phosphorylation of both Akt and GSK3β, but did not significantly alter the levels of water-soluble, detergent-soluble, or formic acid (FA)-soluble forms of Aβ.

MATERIALS AND METHODS

Reagents and antibodies

Unless specified, chemicals were from Sigma-Aldrich (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). Synthetic $A\beta_{1\text{-}42}$ was purchased from the Keck laboratory. $A\beta_{1\text{-}40}$ was synthesized and purified by Dr. James I. Elliott at Yale University (New Haven, CT). Peptide masses and purities were determined by electrospray ionization/ion trap mass spectrometry and reverse-phase HPLC, respectively.

6E10, a monoclonal antibody to residues 1–16 of Aβ, was from Covance. AW8 is a rabbit anti-Aβ polyclonal antibody raised to aggregated synthetic $A\beta_{1\text{-}42}$ and is capable of immunoprecipitating Aβ from culture medium, cerebrospinal fluid and human brain extracts and has been described previously [38]. Simvastatin (SV) was purchased from Molekula Ltd, Shaftesbury, Dorset, UK.

Animals and diet

Double transgenic AβPPswe/PS1dE9 mice and age-matched control littermates bred on a C57BL/6 background were used throughout this study. All experiments were carried out in accordance to guidelines and under license from the Department of Health, Ireland. Experiments were conducted on mice aged 6, 8, or 18 months. Mice were housed in the Conway Institute animal facility with a dark/light cycle of 12 h and fed with chow and water *ad libitum.* Founder wild type C57BL/6 females and heterozygous AβPPswe/PS1dE9 males were obtained from Jackson Laboratories. AβPPswe/PS1dE9 mice have two transgenes (humanized mouse mutant AβPP and PS1) inserted at a single locus under the control of a prion promoter [39, 40]. These mice express a Mo/HuAβPP695swe, transgene allowing the mice to secrete human Aβ peptide. The AβPP Swedish mutation increases the total amount of Aβ produced and the PS1 sequence lacks Exon 9 (dE9) which increases the relative amount of $A\beta_{42}$ compared to A_{40} [39, 40]. To study the effects of chronic SV treatment, AβPPswe/PS1dE9 mice and age-matched control littermates (male and female balanced groups) were fed with chow pellets supplemented with 0.04% SV, representing a daily dose of 400 mg/kg^{-1} of food [41]. A pilot study was conducted in which C57BL/6 mice

were fed with either a control diet or SV supplemented diet. We found that the addition of SV did not alter the mean food intake or body weight between groups (data included in Supplementary Material). At 7 months, our groups of experimental animals were given the supplemented diet for one month. Electrophysiological experiments were then conducted to compare LTP, and extracts of brain tissue were used to examine Aβ content and Akt/GSK-3 levels.

Genotyping procedures

DNA was extracted from ear tissue samples and the presence of transgenes confirmed by PCR. For further details, see Supplementary Material.

Measurement of plasma cholesterol levels

Blood samples were collected immediately following euthanasia, placed on ice, and spun at 3080 g for 10 min at 4°C. Supernatant was collected, frozen in liquid nitrogen, and stored at −80°C for later cholesterol assay. Plasma cholesterol levels were measured using a kit according to the manufacturer's recommendations (Randox Laboratories, Ireland).

Serial extraction of mouse brain tissue for analysis of Aβ

This was done essentially as described previously [31]. The cerebellum and frontal cortex were removed and brain samples frozen in liquid nitrogen and stored at −80°C. Tissue (200 mg) was homogenized with 25 strokes of a Dounce homogeniser (Fisher, Ottawa, Canada) in 5 volumes of tris-buffered saline (TBS) containing ethylene diaminetetraacetic acid (5 mM, EDTA), ethylene glycol tetra-acetic acid (5 mM, EGTA), 10 mg/ml^{-1} leuptin, 1 mg/mg^{-1} pepstatin, and 1 mM Pefabloc. Homogenates were then centrifuged at 176,267 g for 30 min at 4°C in a TLA 100.4 rotor (Beckman Coulter, Fullerton, CA). The supernatant referred to as the TBS extract which contains soluble Aβ species was removed and stored at −80°C. The TBS insoluble pellet was resuspended in TBS containing 1% Triton-X 100 (TBS-TX) plus protease inhibitors, and then homogenized and centrifuged as before. The TBS-TX supernatant was removed, aliquoted, and stored at −80°C. The Triton insoluble pellet was re-suspended in 88% FA (1:0.1 weight/volume), sonicated for 5 min, agitated overnight at 4°C, and then stored at −80°C.

Aβ detection by immunoprecipitation/western blotting

TBS and TBS-TX extracts (500 µl) were diluted 1:1 in TBS to a final volume of 1 ml for immunoprecipitation. Immunoprecipitation samples (TBS and TBS-TX) were pre-cleared with 25 µl of protein A beads sepharose (Sigma) for 1 h at 4°C. Homogenates were then spun at 825 g for 10 min. Supernatant was collected and incubated overnight at 4°C on a nutator with the polyclonal antibody AW8 at a dilution 1:80 [38], plus 25 µl of protein A sepharose beads. Antigen-antibody protein A complexes were collected by centrifugation and washed as described previously [31] and the Aβ-AW8 complex liberated from beads by boiling in 2x sample buffer (1.5 M Tris base (pH 8.45), 20% glycerol, 8% SDS, 0.02% phenol red). FA-extract (4 µl) was allowed to dry at room temperature for 2 days to evaporate the FA. Sample buffer (11 µl) was added to the dried FA extract prior to western blot. Samples were electrophoresed on 10–20% polyacrylamide tris-tricine gels (Invitrogen, Carlsbad, CA, USA) and proteins transferred to 0.2 µm nitrocellulose membrane (Optitran, Scheilcher and Schűll, Germany) at 400 mA for 2 h. To improve Aβ detection, membranes were microwaved for 1.5 min in phosphate buffer saline (PBS) and after 3.5 min turned and microwaved again. Filters were blocked in TBS containing 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) and then washed in TBS containing 0.05% Tween 20 (TBS-T) 4 × 15 min. Filters were incubated with 6E10 (1:1,000) overnight at 4°C washed four times in TBS-T and incubated with fluorochrome-coupled anti-mouse secondary antibody (1:2,500), (Rockland, Gilbertsville, PA, USA). Bound anti-body was detected using a Li-COR Odyssey near infrared imaging system (Li-COR Biosciences, Lincoln, NE, USA). Aβ levels were calculated by reference to known quantities of synthetic $A\beta_{1\text{-}42}$ (20, 10, 5 ng) electrophoresed on the same gel [38]. Average values were obtained from duplicate samples. With regard to controlling for efficient electrotransfer of proteins, we were careful to stain membranes with Ponceau S (prior to immunoblotting) so as to exclude any samples or blots that did not evidence even transfer of proteins.

Akt, GSK3, and tau western blots

For each cortical sample, 200 mg of tissue was homogenized in 1 ml of ice-cold lysis buffer (10 mM Tris HCL, containing 1% Triton X-100, 5 mM EDTA,

5 mM EGTA, 1% SDS, 1% NP-40, 10 mM deoxycholate, 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride (PMSF) and protease and phosphatase inhibitors from Sigma) as described above. Samples were then spun at 9167 g for 5 min at 4°C. Supernatant was collected and the protein concentrations measured using a BCA protein assay (Thermo Scientific, Rockford, IL, USA). Samples (30 μg total proteins) were electrophoresed on 10–20% polyacrylamide tris-tricine gels (Invitrogen) and transferred onto 0.2 μm nitrocellulose membrane (as described above). Membranes were washed in TBS-T and blocked with 5% BSA in TSB-T for 1 h at room temperature. Membranes were then incubated with either rabbit anti-phospho Ser473-Akt antibody (1:1,000, Cell Signaling, MA, USA), rabbit anti-phospho Ser9-GSK3β or rabbit anti-p-Tau (Ser400/Thr403/Ser404) (1:1,000, Cell Signaling). After three washes, membranes were incubated with a secondary anti-rabbit antibody conjugated with horseradish peroxydase (1:2,000 in 5% BSA-TBS-T). Membranes were thoroughly washed in TBS-T and bound antibody detected using ECL and film. To detect total Akt, GSK3, or tau, membranes were stripped blocked and incubated with the corresponding antibody of choice (rabbit anti-total Akt antibody, 1:2,000, or rabbit anti-total GSK3 antibody, 1:2,000 or mouse anti-Tau (Tau 46) mAb, Cell Signaling). Membranes were washed and incubated with an anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase (1:4,000) and bound antibody detected using ECL and film. To measure the level of tubulin (for GSK and AKT) or GAPDH (for tau), membranes were stripped a second time and probed with α-tubulin anti-mouse antibody (1:10,000, Cedar, USA) or anti-mouse GAPDH antibody (Cell Signaling) All films were scanned and analyzed using Image J.

Electrophysiology

Electrodes were pulled from borosilicate capillary glass (GC150 F-10, Harvard Apparatus), using a horizontal puller (DMZ universal puller, Germany). Electrodes (2–5MΩ) were filled with artificial cerebrospinal fluid (NaCl 119 mM; D-glucose 11 mM; $NaHCO_3$ 26 mM; KCl 2.5 mM; $MgSO_4$ 1 mM; $CaCl_2$ 2.5 mM; NaH_2PO_4 1 mM). The voltage signal was filtered at 5 kHz and stored for off-line analysis using a personal computer interfaced with a CED/National Instruments A/D board and WinCP software (J. Dempster, Strathclyde University). The Shaffer-collateral pathway was stimulated using a monopolar electrode (FHC, Bowdoin, USA) at 0.033 Hz (duration: 100 μs), the return electrode was a silver/silver chloride wire placed in the recording bath. Extracellular field recordings were made from the *stratum radiatum* of the CA1 at 30°C. Signals were amplified by a HS2A headstage (Molecular Devices, USA) connected to an Axoclamp 2B system (Molecular Devices) and a Brownlee 410 Precision preamplifier. A Master 8 (AMPI) timer was used to deliver and time the stimulus trigger. Stable field excitatory postsynaptic potentials (EPSPs) were recorded for 20 min, at 40–50% maximum response prior to the application of high frequency stimulation (HFS) to induce LTP. LTP was induced using two trains of stimuli at 100 Hz for 1 s, with an inter-train interval of 30 s. Following the application of HFS, the synaptic response was recorded for a further period of 60 min. Statistical analysis was performed using ANOVA. All results are presented as mean ± SEM.

RESULTS

LTP measurements in AβPPswe/PS1dE9 mice

The magnitude of LTP in the CA1 region measured 60 min following HFS was compared in slices prepared from 6, 8, and 18 month old AβPPswe/PS1dE9 mice and wild type age-matched littermates (controls). There was no significant difference in the magnitude of LTP recorded 55–60 min following HFS in slices taken from 6 month old AβPPswe/PS1dE9 mice ($153.6 \pm 13.6\%$, $n = 9$) and their age-matched controls ($158.2 \pm 13.6\%$, $n = 9$, $p \geq 0.05$) (Fig. 1A).

The magnitude of LTP was significantly reduced in hippocampal slices from 8 month old AβPPswe/PS1dE9 mice ($124.0 \pm 7.7\%$, $n = 6$) compared to controls ($182.7 \pm 16.4\%$, $n = 9$, $p \leq 0.05$) (Fig. 1B). This was also significantly lower than LTP recorded at 6 months in AβPPswe/PS1dE9 slices ($p \leq 0.05$). LTP magnitude was also significantly reduced in hippocampal slices taken from 18 month old AβPPswe/PS1dE9 mice ($113.6 \pm 7.4\%$, $n = 6$) compared to age matched controls ($176.4 \pm 6.4\%$, $n = 10$, $p \leq 0.001$, Fig. 1C). There was no significant difference in the degree of attenuation of LTP recorded in hippocampal slices from 8 and 18 month old AβPPswe/PS1dE9 mice (see summary bar chart summarizing LTP recorded in wild type and AβPPswe/PS1dE9 mice at 6, 8, and 18 months: Fig. 1D).

Aβ levels at 6, 8, and 18 months in AβPP/PS1 mice

To determine if the deficits in LTP recorded at 8 and 18 months were related to an increase in the

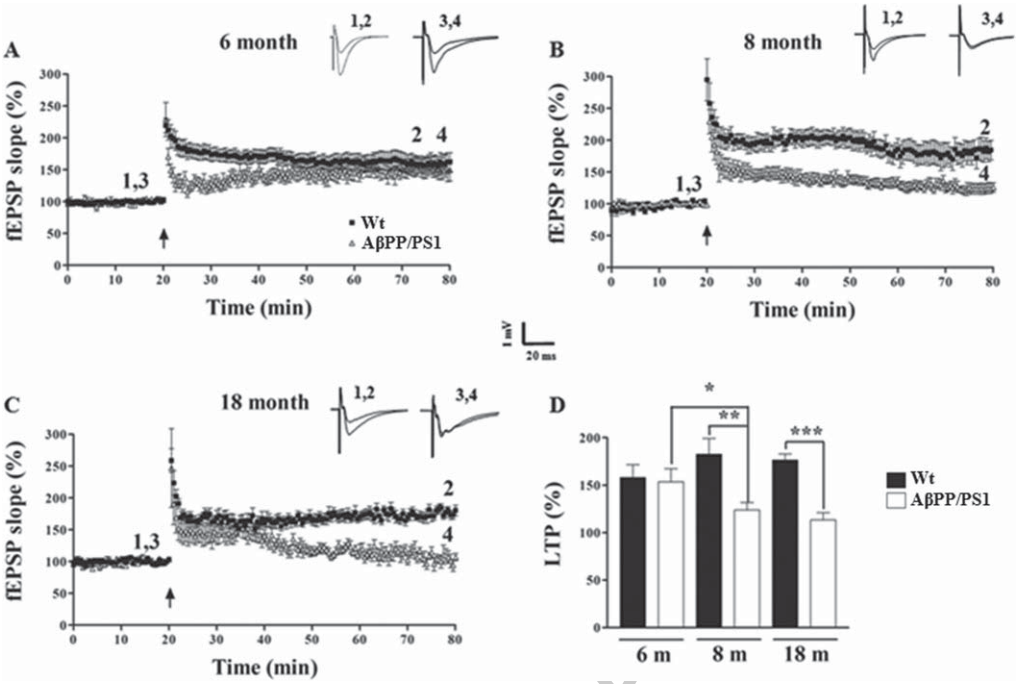

Fig. 1. Age-dependent deficits in LTP in hippocampal slices from AβPPswe/PS1dE9 mice. LTP measurements were performed at 55–60 min post high frequency stimulation. Arrows represent HFS application. Example field excitatory postsynaptic potentials (fEPSPs) are shown above each graph, recorded prior to and following LTP induction at the times indicated on the graphs. A) LTP in slices from 6 month old AβPPswe/PS1dE9 ($n = 9$), was similar to age-matched wild type (Wt) littermates ($n = 9$). B) LTP was impaired in AβPPswe/PS1dE9 hippocampal slices at 8 months ($n = 6$) compared to age-matched Wt littermates ($n = 9$, $p < 0.05$). C) Slices from 18 month old AβPPswe/PS1dE9 ($n = 6$) had a deficit in LTP compared to Wt littermates ($n = 10$, $p < 0.05$). D) Bar charts summarizing LTP in Wt and AβPPswe/PS1dE9 mice at 6, 8, and 18 months.

brain content of Aβ, we examined levels of water-soluble, membrane-bound, and FA-soluble Aβ species across the three age groups. A prominent Aβ monomer band migrating at ∼4 kDa was detected in all samples from AβPPswe/PS1dE9, but not in those from wild type mice (Fig. 2A). An additional more intense band migrating ∼12 kDa was detected in the TBS-TX extracts from transgenic brain, but was not present in TBS or FA extracts from those brains or in TBS-TX extract from wild type mice (Fig. 2A, D). Since the epitope of the western blotting antibody, 6E10, lies between residues 6 and 10 of Aβ, this ∼12 kDa band likely represents C99. The concentration of Aβ detected in TBS brain extract increased on average 8-fold between 6 and 8 months with values for 6 months old of 7.65 ± 1.22 ($n = 6$) and for 8 month old mice 56.51 ± 12.96 ($n = 6$, $p < 0.05$, Fig. 2B). The level of Aβ in 18 month old AβPPswe/PS1sE9 mice was also significantly higher than in 6 month old mice, but although not significantly different from that in 8 month old animals, it tended to be lower (41.09 ± 2.96, $n = 6$ $p \geq 0.05$) (Aβ is expressed in ng/g of wet brain). The levels of Aβ detected in the TBS-TX extract were comparable to those detected in the TBS extract and steadily increased with age, 6 months (14.09 ± 2.98, $n = 6$), 8 months (51.22 ± 2.98, $n = 5$), and 18 months (90.09 ± 6.9, $n = 5$) (Aβ is expressed in ng/g of wet brain, $p \leq 0.01$ for 6 month versus 8 month and $p \leq 0.05$ for 8 month versus 18 month, Fig. 2C). Western blot analysis of FA extracts from transgenic mice revealed a prominent ∼4 kDa band and a light smear of immunoreactive material stretching from this band up to the top of the gel (Fig. 2D). Since the intensity of the smear always correlated with that of the ∼4 kDa band and the latter was more intense than the smear, we based our quantification of Aβ solely on the intensity of the ∼4 kDa band. The level of Aβ in FA extracts was an order of magnitude larger than those in either the TBS or TBS-TX extracts and steadily increased with age reaching a value of 815 ng of Aβ per gram of wet tissue weight; FA-soluble Aβ species increased significantly between 6 months (135.9 ± 21.4, $n = 5$) and 8 months (384.1 ± 78.8, $n = 6$) (Aβ is expressed in μg/g of wet brain, $p \leq 0.05$) and between 8 and 18 months (815.3 ± 121.7, $n = 6$, $p \leq 0.05$) (Fig. 2E). These results demonstrate that the vast majority of Aβ at the three time points studied is present in water- and detergent-insoluble deposits and that the levels of TBS-

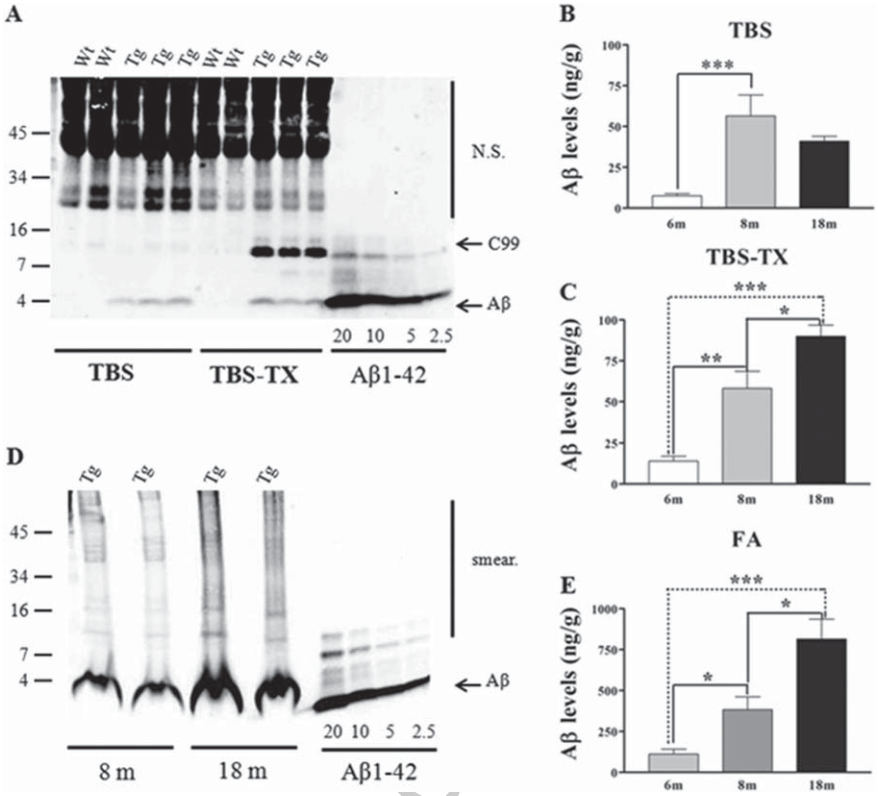

Fig. 2. The concentration of Aβ levels in the water-soluble, detergent-soluble and FA-soluble fractions of AβPPswe/PS1dE9 brain increases with age. A) Example immunoprecipitation/western blot of TBS and TBS-TX fractions from 6 month old wild type (Wt) and AβPPswe/PS1dE9 (Tg) mice. B) Levels of soluble Aβ (TBS extract) increased significantly between 6 and 8 months ($p < 0.001$), but decreased at 18 months, however, this decrease was not significant. C) There was a significant age-dependent increase in the level of membrane bound Aβ in TBS-TX at 6, 8, and 18 months. D) Example western blot showing the age-dependent increase of Aβ in the FA fraction in 8 and 18 month old AβPPswe/PS1dE9 brains. E) The levels of FA-soluble Aβ increased in an aged-dependent manner between 6, 8, and 18 months. For each n, samples were analyzed in duplicate and the average was calculated. $^{*}p = 0.05$, $^{**}p = 0.001$, $^{***}p = 0.0001$. In the bar charts, results are presented as the mean ± SEM.

soluble Aβ reached a plateau while water-insoluble Aβ continued to accumulate.

Chronic administration of SV protects against the age-dependent impairment of LTP in AβPPswe/PS1dE9 mice

As we observed age-dependent deficits in LTP in hippocampal slices from AβPPswe/PS1dE9 mice between 6 and 8 months (Fig. 1), an interval when the levels of Aβ were drastically increased, we investigated the effects of treating mice with SV at this critical time. We examined LTP in slices from AβPPswe/PS1dE9 and wild type littermates (controls) that received a diet supplemented ± SV for one month (month 7) and compared the level of LTP to that recorded in slices from age-matched mice that had received the control diet. Chronic SV treatment had no significant effect on the magnitude of LTP recorded in slices taken from 8 month old control mice ($199.4 \pm 16.9\%$, ($n = 8$), $p \geq 0.05$), compared to age-matched untreated controls ($182.7 \pm 16.4\%$, $n = 9$, $p \geq 0.05$) (Fig. 3A). However, when we examined slices from AβPPswe/PS1dE9 mice that had received the SV-supplemented diet there was a significant increase in the magnitude of LTP, which measured ($191.5 \pm 12.1\%$, $n = 7$) compared to AβPPswe/PS1dE9 mice that had received normal unsupplemented chow ($124.0 \pm 7.7\%$, $n = 6$; $p \leq 0.001$) (Fig. 3B).

Effect of SV on Aβ levels

To determine if the increased levels of LTP recorded following SV treatment resulted due to alterations in Aβ, we quantified Aβ in three biochemically distinct fractions of mouse brain. We found that SV-treatment caused a near 30% decrease in the levels of TBS- and TBS-TX-soluble Aβ, but due to the inherent vari-

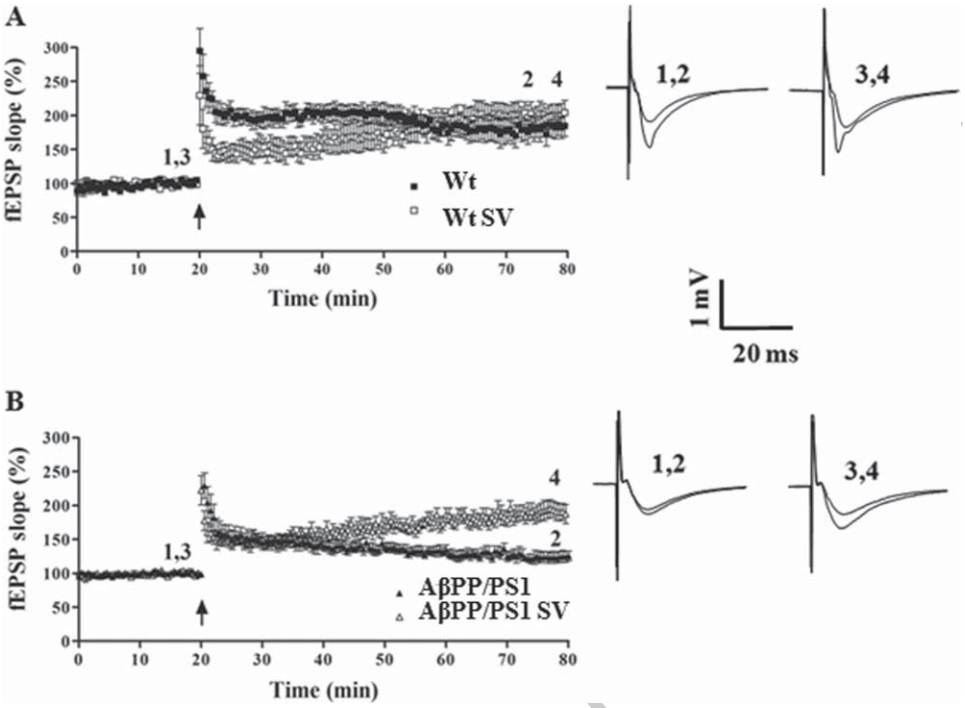

Fig. 3. Chronic SV treatment rescued LTP deficits in slices from 8 month old AβPPswe/PS1dE9 mice. A) Chronic SV had no significant effect on LTP recorded in slices from 8 month old wild type (Wt) mice ($199.4 \pm 16.9\%$, $n = 8$, $p = 0.49$) compared to age matched non-treated wild types ($182.7 \pm 16.4\%$, $n = 9$). B) LTP deficits observed in slices from 8 month AβPPswe/PS1dE9 mice were significantly reduced following one month SV treatment ($191.5 \pm 12.1\%$, $n = 7$) compared to untreated AβPPswe/PS1dE9 ($124.0 \pm 7.7\%$, $n = 6$, $p \leq 0.001$). The magnitude of LTP was similar to age matched control levels in the treated and non-treated conditions in both cases ($p > 0.05$). The black arrow represents time of HFS. Above each set of graphs, representative fEPSPs are shown that were recorded at the times indicated by the numbers on each graph. All values are presented as the mean ± SEM of the fEPSP slope normalized from the baseline. Statistical analysis was performed using an unpaired Students *t*-test.

ability in the levels of Aβ in different animals, this reduction was not statistically significant. SV treatment also had no effect on the levels of the FA-soluble Aβ species (413.4 ± 37.0, $n = 6$) which was similar to the level in the age-matched non treated group (384.1 ± 78.8, $n = 6$, $p \geq 0.05$). Aβ levels in the FA fraction are expressed in ng/g wet brain (Fig 4D).

Akt and GSK3β levels following SV treatment

SV treatment rescued the deficit in LTP recorded in slices obtained from AβPPswe/PS1dE9 mice at 8 months, yet had only a modest effect on Aβ levels, suggesting that the protective effect of SV may be modulated by a process independent of Aβ modulation. Since synapto-toxic forms of Aβ have been shown to reduce phosphorylation of GSK-3-serine 9 [32] we examined the levels GSK-3 and Akt, two key enzymes, involved in synaptic plasticity [33]. In addition, as GSK-3 is known to phosphorylates tau at multiple sites [42], we examined levels of phospho- and total murine tau.

SV treatment did not significantly change the level of p-Akt in the wild type treated group ($110.5 \pm 7.8\%$, $n = 5$) compared to untreated controls ($100.0 \pm 3.5\%$, $n = 6$, $p \geq 0.05$). Of interest however, we observed an increase in the level of p-Akt in the AβPPswe/PS1de9 treated group (112.1 ± 4.0, $n = 4$) compared to the untreated transgenic group (91.8 ± 5.9 %, $n = 6$, $p \leq 0.05$) (Fig. 5A). SV treatment did not alter the level of total Akt in wild type mice ($94.18 \pm 4.4\%$, $n = 5$), which was similar to non-treated controls ($100.0 \pm 2.9\%$, $n = 6$, $p \geq 0.05$). There was also no significant difference between the level of total Akt in SV-treated AβPPswe/PS1dE9 (97.2 ± 3.7, $n = 4$) and non-treated mice ($95.7 \pm 2.4\%$, $n = 6$, $p \geq 0.05$) (Fig. 5B). The ratio of p-Ser473 Akt/total Akt in brain extracts from wild type mice (1.003 ± 0.043, $n = 6$) was similar to SV-treated wild types (1.196 ± 0.132, $n = 5$, $p \geq 0.05$) (Fig. 5C). SV treatment however significantly increased the p-ser473 Akt/total Akt ratio in AβPPswe/PS1dE9 mice (1.158 ± 0.057, $n = 4$) compared to age-matched untreated AβPPswe/PS1dE9 mice (0.960 ± 0.057; $n = 6$, $p \leq 0.05$) (Fig. 5C).

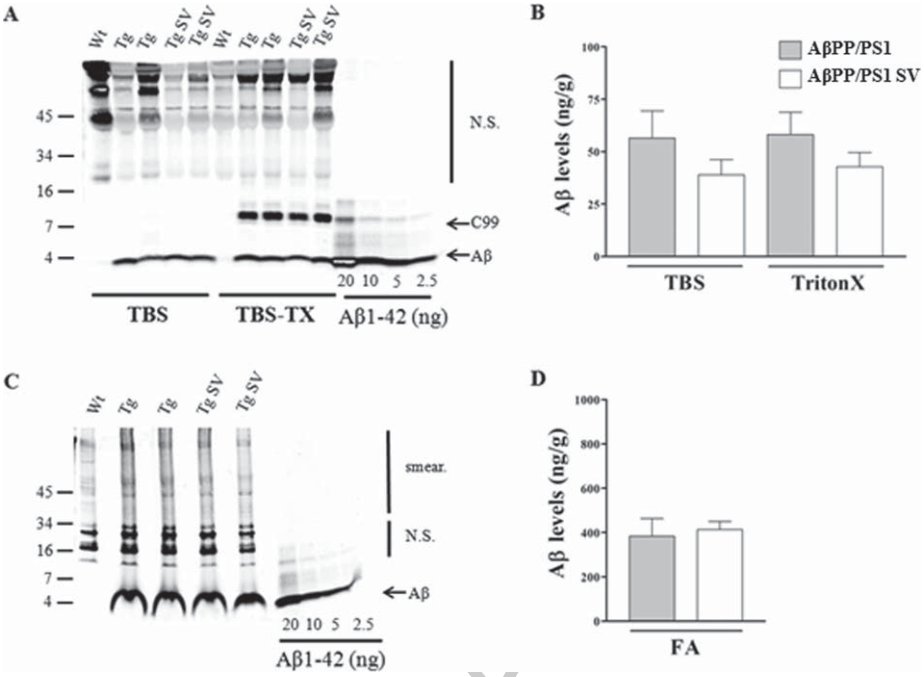

Fig. 4. Chronic SV treatment did not significantly alter cerebral Aβ levels in 8 month old AβPP/PS1 mice. A) Example immunoprecipitation/western blot showing detection of Aβ in the TBS and TBS-TX fractions prepared from 8 month old murine brain (Wt, wild type; Tg, AβPPswe/PS1dE9 mice, and Tg SV, simvastatin treated AβPPswe/PS1dE9 mice). B) Treatment with SV did not significantly alter the levels of TBS-soluble Aβ (39.0 ± 7.3, $n=6$), compared to the untreated cohort (56.5 ± 13.0, $n=6$), $p>0.05$. The level of membrane-bound Aβ in the SV treated group (42.9 ± 6.7, $n=6$), was also not significantly lower that the untreated group (58.3 ± 10.6, $n=6$, $p>0.05$). Values are expressed in ng/g of wet weight brain. C) Example western blot showing detection of Aβ in the FA fraction from 8 month old SV-treated AβPPswe/PS1dE9 and non-treated transgenic brains. D) Aβ levels in the FA fraction were similar in SV-treated (413.4 ± 37.0, $n=6$) and untreated groups (384.1 ± 78.8, $n=6$). All values are represented as the mean ± SEM and expressed in ng/g of wet brain.

The level of p-GSK3 was significantly lower in AβPPswe/PS1dE9 mice ($82.8 \pm 2.2\%$, $n=6$) compared to age-matched wild types ($100.0 \pm 6.7\%$, $n=6$) (Fig. 6A), but this was overcome by SV treatment that caused a significant increase in the level of p-GSK3 in SV-treated AβPPswe/PS1dE9 mice ($119.9 \pm 11.7\%$, $n=4$, $p=0.005$). However, SV had no effect on p-GSK3 levels in wild type mice ($104.1 \pm 4.9\%$, $n=5$, $p \geq 0.05$) (Fig. 6A). In contrast the level of total GSK3 was similar in all four groups; wild type ($100.0 \pm 5.3\%$ ($n=6$); AβPPswe/PS1dE9 ($92.58 \pm 2.1\%$, $n=6$); SV-treated wild type ($89.72 \pm 5.01\%$, $n=5$, $p \geq 0.05$); AβPPswe/PS1dE9 mice ($101.4 \pm 6.5\%$, $n=4$, $p \geq 0.05$) (Fig. 6B). The phospho-GSK3/total GSK3 ratio was increased significantly in SV-treated 8 month old wild type mice (1.166 ± 0.043 ($n=5$) compared to age-matched untreated wild types (0.997 ± 0.017, $n=6$, $p \leq 0.01$) (Fig. 6C). In addition, SV treatment increased significantly the phospho-GSK3/total GSK3 ratio from 0.897 ± 0.035 ($n=6$) to 1.176 ± 0.059 ($n=4$) ($p \leq 0.01$) in extracts from AβPPswe/PS1dE9 mice (Fig. 6C). The ratio of p-GSK3/total GSK3 was significantly lower in the non-treated AβPPswe/PS1dE9 group (0.897 ± 0.035, $n=6$) compared to the non-treated wild type group (0.997 ± 0.016, $n=6$, $p \leq 0.05$). As tau is phosphorylated by GSK3, we examined the level of tau phosphorylation across our treatment groups. We found that although levels of p-tau/tau were not significantly different between any of our groups of animals (see Supplementary Fig. 2).

The effects of SV on serum cholesterol measurements at 8 months

To verify that SV was biologically active, we assayed serum cholesterol levels in mice at the end of the one month treatment period. As expected, treatment with the SV significantly decreased the levels of serum cholesterol in wild type mice (2.02 ± 0.22 mM; $n=5$, $p \leq 0.05$) compared to the untreated control group (2.67 ± 0.15 mM; $n=5$). Likewise SV significantly reduced plasma cholesterol levels in 8 month AβPPswe/PS1dE9 mice (2.04 ± 0.30 mM; $n=5$,

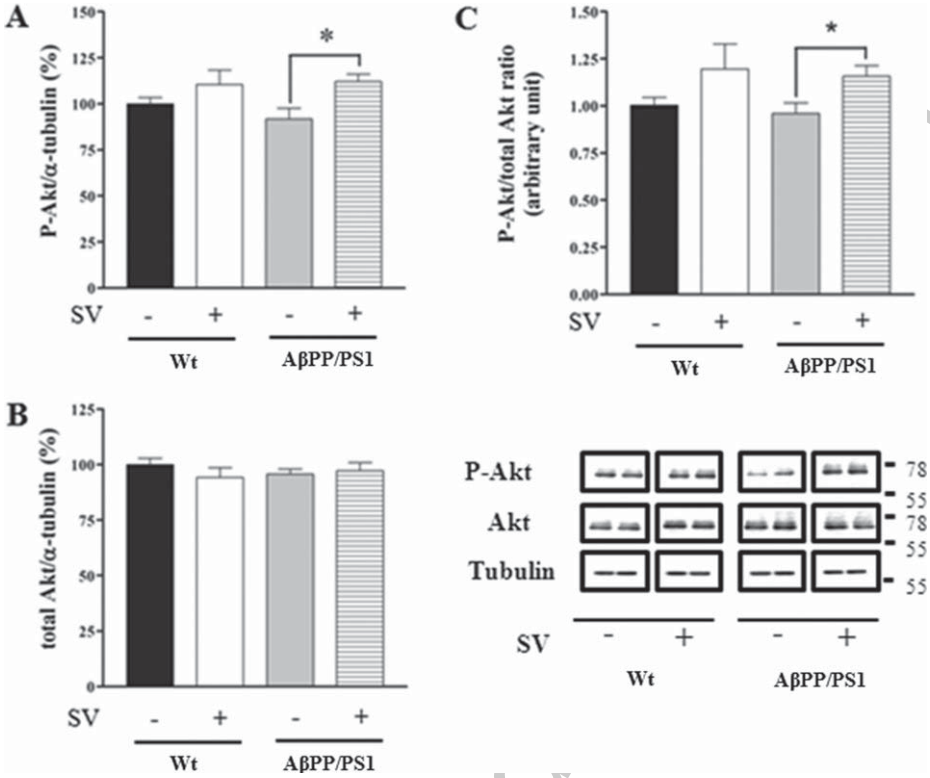

Fig. 5. Chronic SV restores normal levels of activated Akt in brain extracts from AβPPswe/PS1dE9 mice. A) The level of p-Akt was similar in wild type ($100.0 \pm 3.5\%$, $n = 6$) and AβPPswe/PS1dE9 mice ($91.8 \pm 5.9\%$, $n = 6$). SV treatment had no significant effect on the p-Akt levels in wild-type compared to non-treated mice ($110.5 \pm 7.8\%$, $n = 5$, $p = 0.22$). P-Akt levels were increased significantly in SV-treated AβPPswe/PS1dE9 mice compared to the non-treated group ($112.1 \pm 4.0\%$, $n = 4$, $p = 0.03$). B) There was no change in the level of total Akt across the control, transgenic and SV-treated groups. C) The p-Akt/total Akt ratio in SV-treated wild type mice (1.196 ± 0.132, $n = 5$) was similar to non-treated mice (1.003 ± 0.043, $n = 6$. $p = 0.17$). SV-treated AβPPswe/PS1dE9 mice had a significantly higher p-Akt/total Akt ratio (1.158 ± 0.057, $n = 4$) compared to the non-treated transgenic group (0.960 ± 0.057; $n = 6$, $p \leq 0.05$).

$p \leq 0.05$) compared to untreated AβPPswe/PS1dE9 mice (3.04 ± 0.30 mM; $n = 6$) (Supplementary Fig. 1).

DISCUSSION

In this study we have investigated the correlation between altered synaptic plasticity and Aβ load in the AβPPswe/PS1dE9 mouse model of AD. In addition, we have investigated the possible beneficial effects of SV administration on Aβ content and synaptic plasticity at a critical time in AβPPswe/PS1dE9 mice. Due to the pathological features of AD, which include abnormal accumulation of neurotoxic Aβ plaques within the brain [43], models used to study AD have included acute application of Aβ peptide to the hippocampus *in vivo* [44] and *in vitro* [45]. Transgenic mouse models which slowly accumulate increasing concentrations of Aβ have become increasingly popular [46]. In this study, we used the AβPPswe/PS1dE9 mouse model which overexpresses both the Swedish mutation of AβPP and mutant PS1 deleted in Exon 9, both mutations are linked to familial inherited forms of AD [47]. These mice have now been studied by many groups and are known to develop Aβ plaques at 4 months accompanied by plaque-associated activated microglia and astrocytes. Spatial navigation and reference learning deficits have been reported using the radial arm water maze at 12 months [48]. These mice also have neuritic abnormalities at 7–8 months [49]. In addition, several groups have also studied hippocampal LTP in this mouse model [50, 51]. While one group reported a lack of LTP deficit [51], they also reported much lower levels of Aβ.

Cognitive decline and memory deficits associated with AD are linked to synaptic neuronal network dysfunction and ultimately neuronal degeneration. Aβ is known to cause a deterioration of the synaptic function linked to decreased synaptic plasticity, and recent evidence also demonstrates alterations of the intrinsic

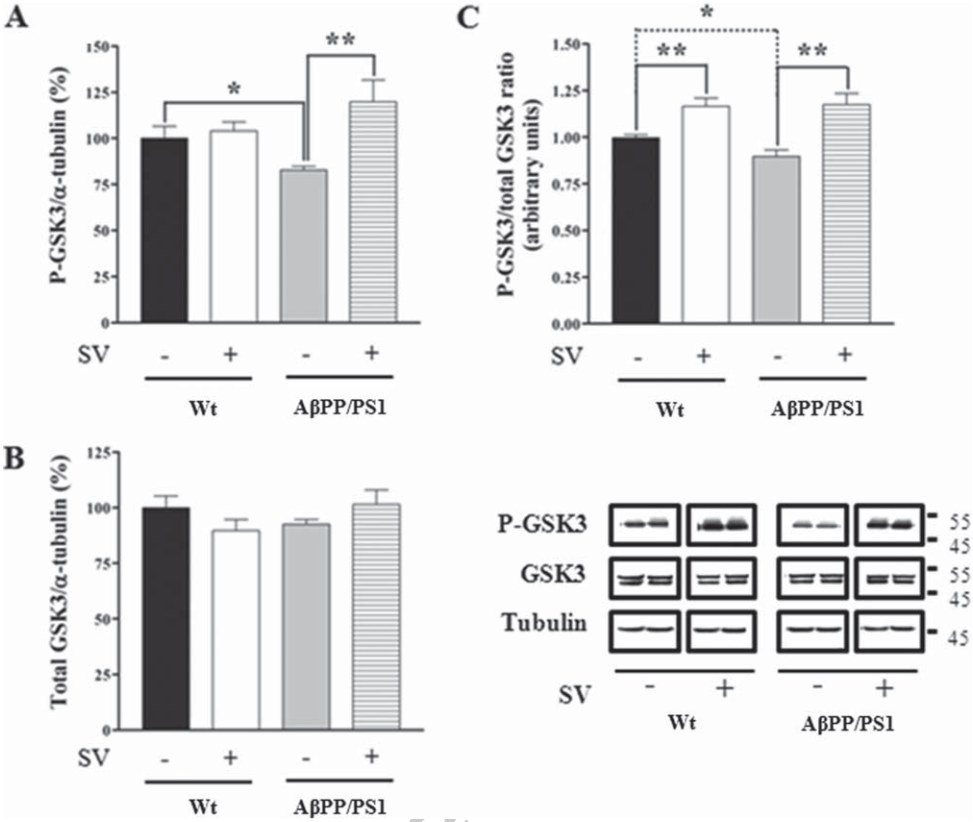

Fig. 6. SV treatment restores activation of GSK3 in AβPPswe/PS1dE9 mice. A) p-GSK3 levels were significantly lower in brain homogenates from 8 month old AβPPswe/PS1dE9 mice ($82.8 \pm 2.2\%$, $n = 6$) compared to age matched wild types ($100.0 \pm 6.7\%$, $n = 6$, $p \leq 0.05$). SV treatment had no significant effect on the p-GSK3 levels in wild type mice ($104.1 \pm 4.9\%$, $n = 5$) compared to untreated wild type mice. The level of p-GSK3 in SV-treated AβPPswe/PS1dE9 mice was significantly increased ($119.9 \pm 11.7\%$, $n = 4$, $p \leq 0.005$). B) Levels of total GSK3 were similar in all groups. C) The p-GSK3/totalGSK3 ratio was significantly lower in AβPPswe/PS1dE9 mice (0.897 ± 0.035, $n = 6$) compared to age-matched wild types (0.997 ± 0.016, $n = 6$, $p \leq 0.05$). The p-GSK3/total GSK3 ratio was significantly increased in SV-treated AβPPswe/PS1dE9 mice (1.176 ± 0.059, $n = 4$; $p \leq 0.005$) and SV treated wild types (1.166 ± 0.043, $n = 5$; $p \leq 0.005$) compared to untreated mice. The ratio of p-GSK3/total GSK3 was similar between SV-treated AβPPswe/PS1dE9 and SV-treated wild types.

excitability of neurons in AD mouse models [52, 53]. In our study, we focused on synaptic plasticity in the form of hippocampal LTP, a well-documented cellular model of learning [54]. In view of the amyloid cascade hypothesis [16, 17], we examined the levels of soluble, membrane bound, and FA-soluble Aβ species, to determine if there was a correlation between any observed alterations in LTP and Aβ load. Having established the characteristics of our model, we tested the effects of chronic administration of SV, an agent which has been proposed to be protective against AD [7, 8, 12].

LTP and Aβ load

Our results show a clear age-dependent impairment of LTP in hippocampal slices from AβPPswe/PS1dE9 mice. We did not observe any impairment in LTP in slices from 6 month old mice, consistent with a previous report [51]. However, in slices from 8 month old AβPPswe/PS1dE9 mice, we observed a significant deficit in LTP which was sustained in slices from 18 month old animals. Increased levels of soluble Aβ have also been shown to be associated with deficits in spatial learning and memory at 12 months in AβPPswe/PS1dE9 mice [55]. Our observed attenuation in LTP supports the learning deficits previously reported in these mice between 8 and 18 months [48, 53, 55, 56]. Most ELISAs appear to preferentially detect Aβ monomer [57, 58]; the use of such assays would not detect Aβ oligomers. Therefore we employed an immunoprecipitation/western blot assay which can capture both oligomeric and monomeric Aβ [59]. Analysis of the AβPP/PS1 mouse brains used in this study indicates that the water-soluble phase (TBS extract) contained Aβ species which migrated on SDS-PAGE as a ∼4 kDa monomer. The gels used are

highly denaturing, thus the ∼4 kDa species detected may not necessarily reflect native Aβ assembly size. Because fibrils are removed by centrifugation, the species detected on SDS-PAGE are unlikely to be SDS-induced breakdown products of fibrils. Thus the ∼4 kDa species detected on SDS-PAGE could be a true monomer and/or monomer derived from pre-fibrillar assemblies that are unstable when electrophoresed in SDS.

In our study, we quantified Aβ levels in the soluble, membrane associated, and FA fractions of 6, 8, and 18 month old AβPPswe/PS1dE9 mice. We also found a direct association between the age-dependent deficit in LTP and all forms of Aβ. The observed deficit in LTP between 6 and 8 months was accompanied by a highly significant increase in the level of Aβ in the TBS, TBS-TX, and FA fractions. The level of Aβ detected in the TBS and TBS-TX extracts from 6 month old mice is at the limit of detection of the western blotting system used and was below the level of the lowest standard (Fig. 2A). Therefore the estimated Aβ concentration in these extracts may not be highly accurate. However, the level of Aβ detected in extracts from the 8 and 18 month mice were always higher than the lowest standard and therefore their values are reliable. Moreover, absolute accuracy of the values for the 6 month old mice does not detract from the observation that the levels of Aβ increase dramatically in AβPP/PS1 mice between 8 and 8 months.

Variations in reported Aβ load in AβPPswe/PS1dE9 mice

There are variations in the reported levels of Aβ in this mouse model [51, 53, 56]. The levels of Aβ we detected are similar to those reported previously [56], demonstrating total Aβ levels at 7 months to be in the region of 25 nM, approximately 100 ng/g, increasing to 220 nM at 19 months (∼880 ng/g); similar to the total levels we determined. In one study, which found no age-dependent reduction in LTP [51], the levels of cortical Aβ appeared to be substantially lower than those reported in our study. This difference in Aβ content may explain why we observed an age-dependent and Aβ-dependent attenuation of LTP.

Effects of chronic SV treatment

The dose of SV used in our study is high compared to the maximum dose which is approved by the U.S Food and Drug Administration for human treatment; 40 mg /day. The dose we have used is similar to that used in other murine studies in which SV has been administered for periods of up to three months [4]. High doses of SV have been associated with renal failure in humans, however as reported [4], SV (50 mg/kg body weight) did not alter levels of mouse plasma transaminase, which is a marker of hepato-toxicity. It should be noted that in our study we have used high doses to investigate the effect of statin treatment at a critical time of amyloid production and deposition (7–8 months) in our mouse model.

Epidemiological studies suggest that SV reduces the risk of developing dementia and AD [7, 8, 12, 60], however there is controversy which may relate to blood-brain barrier permeability and the stage of AD at which statin is administered [61]. Cholesterol dysregulation is now associated with many forms of neurodegeneration [62]. High cholesterol levels in midlife are reported to be a risk factor for the development of AD [63]. *In vitro* studies suggest that high cholesterol levels support amyloidogenic processing of AβPP [21, 64, 65]. Cholesterol depletion can also reduce Aβ production in hippocampal neurons [21]. SV can also alter the association of the NMDAR1 subunit with lipid rafts [66], thereby altering the potential for calcium influx via activation of this receptor-channel complex. This may reduce neurotoxicity in the event of increased extracellular glutamate which is a proposed mechanism for Aβ-mediated LTD [67]. Statin treatment has also been shown to reduce Aβ-mediated production of pro-inflammatory cytokines, e.g., IL-1β [68], and to increase the production of anti-inflammatory cytokines, e.g., IL-4 [69].

The observed decrease in plasma cholesterol following SV treatment (see Supplementary Material) verified that this statin had biological activity; plasma cholesterol levels were consistent with those reported previously [4]. The dose of SV used in our study has previously been shown to enhance learning and memory in behavioral tasks in both wild type and Tg2576 mice [4]. In another study, however, SV did not alter cognition in adult or aged wild type mice [41]. Following treatment for one month with SV, we did not observe any change in LTP in wild type slices (Fig. 3A). However, the LTP impairments observed in slices at 8 months from AβPPswe/PS1dE9 mice were reversed following SV treatment (Fig. 3B). This result suggested that SV may have either reduced levels of soluble Aβ and/or attenuated the cellular processes whereby Aβ disrupts synaptic plasticity. Acute application of SV has been shown to enhance LTP *in vitro* [70], possibly via inhibition of farnesylation [71]. We did not, however, observe any enhancement of LTP in

control mice following chronic SV treatment.

In our study, SV treatment caused a reduction in soluble TBS-Aβ and membrane bound, TBS-TX Aβ content at 8 months, however, the decrease was not statistically significant. This may have been in part due to the variability between mice for soluble and membrane associated Aβ. As we used the monoclonal antibody 6E10, which recognizes residues 1 to 16 within the Aβ sequence, we could not determine if there was a change in the ratio of $A\beta_{1\text{-}40/1\text{-}42}$ [72]. $A\beta_{1\text{-}42}$ is known to be more prone to aggregation and fibril formation [73] and is more toxic than $A\beta_{1\text{-}40}$, with small changes in the $A\beta_{42/40}$ influencing neurotoxicity [74]. SV may have reduced the $A\beta_{42/40}$ ratio and/or decreased the Aβ induced neurotoxicity. SV treatment also had no effect on levels of FA soluble Aβ. In agreement with our observations, a recent study using J20 mice reported that SV had no effect on brain amyloidosis [41].

Aβ signaling, Akt, and GSK3

Aβ behaves as an antagonist of the insulin receptor, preventing the activation of PI3 kinase, and subsequently phosphorylation of Akt [75]. Mechanistically, Akt (PKB) can inactivate GSK3 by phosphorylation at Ser21 in the case of GSK3α or at Ser9 (GSK3β) [76]. We therefore investigated the levels of activated (phosphorylated) p-Akt following SV treatment. Consistent with a previous report [4], we observed an increase in p-Akt following SV treatment, however this was only significant in AβPPswe/PS1dE9 mice (Fig. 5A). While SV did not alter total Akt levels, it caused a significant increase in the ratio of p-Akt/total. This alteration would favor conditions under which LTP can be induced [77]. This observation is also consistent with the increase in p-GSK that we observed following SV treatment. It is interesting that SV caused an increase in the level of p-GSK in both wild type and transgenic mice, yet there was no overall change in LTP levels in wild type treated mice. This observation is consistent with a report which demonstrated that inhibition of GSK-3 does not alter LTP in control hippocampal slices but blocks the attenuation of LTP caused by Aβ [32]. Aβ is likely to alter phosphorylation of GSK as reported recently [32]. Synthetic $A\beta_{42}$-derived diffusible ligands in the high nanomolar range can impair LTP by activation of caspase-3, promoting GSK3β activation (reduced phosphorylation) via an Akt1 cleavage dependent mechanism [32], likely promoting LTD [33]. In our study, basal levels of phosphorylated GSK3β in brain homogenates from mice at 8 months were significantly lower in AβPPswe/PS1dE9 compared to age-matched non-transgenic littermates (Fig. 6A). It is therefore feasible that increased levels of cerebral Aβ caused a significant decrease in p-GSK3β. This attenuation in p-GSK3 levels may have a major impact on synaptic plasticity, as GSK3 activity has been reported to play a pivotal role in the inhibition of LTP subsequent to induction of LTD [33]. In agreement with the link between p-GSK and LTP, our data support the fact that increased levels of unphosphorylated GSK3 would tend to favor LTD in AβPPswe/PS1dE9 mice. While NMDA receptor dependent calcium influx is linked to LTD [33, 78], other mechanisms including alterations in glutamate uptake are also thought to be responsible for Aβ-mediated LTD [67].

GSK3β in AD

GSK3 can regulate Aβ-degradation by matrix metalloproteases MMP2 and MMP3 in a PI3K dependent manner in AβPP-CHO cells cultures [79]. In blood samples from AD patients and individuals diagnosed with mild cognitive deficits, GSK3α/3β protein levels and GSK3β activity in white blood cells is increased, while the Ser9-phosphorylated GSK-3β levels were decreased compared with healthy age-matched control subjects [80]. GSK3 can phosphorylate 17 of the serine and 6 of the threonine residues of tau [42, 81]. In addition, GSK3 polymorphism has been linked to sporadic AD [82]. Finally, it is well accepted that tau is abnormally hyperphosphorylated in the AD brain [83, 84] leading to the formation of neurofibrillary tangles that are a well-documented hallmark of AD. We also examined levels of tau and tau phosphorylation across our groups of mice. As we probed using the antibody to p-tau (Ser 400/Thr403/Ser404), we would have predicted that increased levels of p-GSK3β, decreasing activity of this enzyme would have caused a decrease in p-tau in the SV treated groups. Sites Ser400 and Ser404 are known to be phosphorylated by GSK3 [42]. While alteration in p-GSK and p-Akt may be beneficial in rescuing LTP, the multiple pleiotropic effects of statins must also be considered, including reduced levels of inflammatory cytokines [68] and increased cerebrovascular reactivity and basal endothelial nitric oxide synthesis [41]. Our data demonstrate that chronic SV treatment in an AD mouse model can rescue deficits in synaptic plasticity in a manner that is independent of total Aβ load. A potential therapeutic mechanism could be via increased GSK3 phosphorylation through the PI3K/Akt pathway. Considering the mounting evidence for a role of GSK3 in AD and increased levels in

the prodromal phase of this disease [72], SV therapy at a critical time could prove to be highly beneficial.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1950).

SUPPLEMENTARY MATERIAL

Supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-130257.

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