Lithium, a Common Drug for Bipolar Disorder Treatment, Regulates Amyloid-β Precursor Protein Processing

Yuan Su,*,‡ John Ryder,‡,# Baolin Li,‡ Xin Wu,‡ Niles Fox,‡ Pat Solenberg,‡ Kellie Brune,‡ Steven Paul,‡,§,⊥ Yan Zhou, Feng Liu, and Binhui Ni*,‡,§,||

Lilly Research Laboratories, Indianapolis, Indiana 46285, and Departments of Psychiatry, Anatomy and Cell Biology, and Pharmacology, Indiana University Medical School, Indianapolis, Indiana 46202

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ABSTRACT: Lithium is one of the most widely used mood-stabilizing agents for the treatment of bipolar disorder. Although the underlying mechanism(s) of this mood stabilizer remains controversial, recent evidence linking lithium to neurotrophic/neuroprotective effects (Choi and Sung (2000) *1475*, 225−230; Davies et al. (2000) *351*, 95−105) suggests novel benefits of this drug in addition to mood stabilization. Here, we report that both lithium as well as valproic acid (VPA) inhibit β-amyloid peptide (Aβ) production in HEK293 cells stably transfected with Swedish amyloid precursor protein $(APP)_{751}$ and in the brains of the PDAPP (APP_{V717F}) Alzheimer's disease transgenic mouse model at clinically relevant plasma concentrations. Both lithium and VPA are known to be glycogen synthase kinase-3 (GSK3) inhibitors. Our studies reveal that GSK3β is a potential downstream kinase, which modulates APP processing because inhibition of GSK3 activity by either a dominant negative GSK3β kinase-deficient construct or GSK3β antisense oligonucleotide mimics lithium and VPA effects. Moreover, lithium treatment abolished GSK3β-mediated Aβ increase in the brains of GSK3β transgenics and reduced plaque burden in the brains of the PDAPP (APP_{V717F}) transgenic mice.

β amyloid (Aβ)[1] is a 39−43 amino acid fragment derived from defined proteolysis of the amyloid precursor protein (APP) and is a major component of senile plaques (*1*, *2*). Aβ is secreted constitutively by normal cells, such as HEK293 cells, in culture and is circulated in plasma and cerebrospinal fluid (CSF) of healthy humans and other mammals (*3*). APP is subjected to proteolytic processing by a series of proteases known as secretases. The cleavage of APP through β- and γ-secretases generates Aβ and soluble APP_β ($sAPP_\beta$), along an amyloidogenic pathway (*4*). It has been reported that the γ-secretase complex contains a variety of proteins including presenilin (*5*−*7*), a known Alzheimer's disease (AD) causing gene, Aph-1, Pen-2, and Nicastrin (*8*). Recent studies have shown that glycogen synthase kinase-3β (GSK3β) interacts with and binds presenilin in normal human brains and cells (*9*, *10*). Although GSK3 is known to be involved in τ phosphorylation, its role in APP processing is poorly understood.

Lithium remains the most widely used treatment for bipolar disorder and represents one of the most important interventions of psychiatry. Despite demonstrated efficacy in reducing both the frequency and severity of recurrent affective episodes and decades of clinical use, the molecular mechanism(s) underlying the therapeutic actions of lithium has not been fully elucidated (*11*). Recently, extensive studies in cells or in vivo have clearly documented that several of the prominent biochemical effects of lithium, such as inhibition of the phosphorylation of the microtubule-associated protein τ, increased levels of β catenin, and protection from GSK3β-facilitated apoptosis, are directly dependent on lithium's inhibition of GSK3β (*12*−*14*), suggesting that lithium is an inhibitor of GSK3β (*15*, *16*). These findings are substantiated by a recent study of 24 kinases showing GSK3β and closely related GSK3α to be kinases substantially inhibited by lithium (*17*), although, CK2, PRAK, and MAPKAP-K2 were also inhibited by lithium, albeit less potently. Further and interestingly, valproic acid (VPA), another commonly used mood stabilizer, has also been recently shown to be a GSK3β inhibitor (*18*). Indeed, several hypotheses have been offered to explain the action of lithium. The most popular being that lithium acts through uncompetitive inhibition of inositol monophosphatase leading to inositol depletion (*19*, *20*). This hypothesis, however, is not supported by inositol monophosphate inhibition data that demonstrate no correlative effect to that of lithium's on the morphogenesis of *Xenopus* embryos (*21*). In this paper, we used lithium as well as VPA and molecular biology tools including a GSK3β kinase-deficient construct, GSK3β antisense oligonucleotide (ASO), and a GSK3β transgenic to investigate whether GSK3β activity is involved in APP processing observed in HEK293 cells and in the brains of PDAPP (APP_{V717F}) transgenic mice.

* To whom correspondence should be addressed: Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285. Telephone: 317-276-9491. Fax: 317-433-7741. E-mail: su_yuan@lilly.com (Y.S.); binhuini1@yahoo.com (B.N.).

‡ Lilly Research Laboratories.

§ Department of Psychiatry.

|| Department of Anatomy and Cell Biology.

⊥ Department of Pharmacology.

# The author contributed equally to the paper.

[1] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, β-amyloid peptide; GSK3, glycogen synthase kinase-3; HEK, human embryonic kidney; sAPP, soluble APP; $swAPP_{751}$, APP_{751} bearing the Swedish mutation; $wtAPP_{751}$, wild-type APP_{751}.

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EXPERIMENTAL PROCEDURES

In Vivo Studies: Acute Lithium or VPA Treatment of PDAPP Mice. All animal studies described in this paper were carried out under the guidelines and approval of the Eli Lilly and Company Animal Care and Use Committee. Two-to-three-month-old PDAPP (APP_{V717F}) transgenic mice were used in these studies. For lithium exposure, mice were randomly divided into two groups with each group containing 10 animals. Mice received an oral dose of 300 mg/kg NaCl or 300 or 600 mg/kg LiCl. For the VPA study, as with the lithium treatment, mice were randomly divided into two groups each containing 10 animals. In the first group, mice received an oral dose of water as a control, while group two mice received an oral dose of 400 mg/kg VPA. A total of 3, 12, and 15 h post dosing, mice were CO_2 anesthetized, blood samples were taken via cardiac puncture into EDTA-treated eppendorf tubes for [Li^+] or [VPA] plasma measurement, and mice were decapitated. Upon brain dissection, hippocampus tissue was immediately homogenized in a 5 M guanidine and 50 mM Tris-HCl at pH 8.0 solution (*22*) and Aβ levels were assessed using an Aβ sandwich ELISA.

Chronic Lithium Treatment of PDAPP Mice. For chronic in vivo studies, 4-week-old PDAPP (APP_{V717F}) mice were fed with a lithium carbonate diet (2.4g/kg, product F3745) or a base diet (product F3028) for 7 months. Lithium carbonate and control base diet food pellets were prepared by BIO-SERV (Frenchtown, NJ) as described previously (*23*). Blood samples via cardiac puncture were collected at the time of harvest to determine the [Li^+] in plasma. Again, following CO_2 anesthetizing, hippocampus was harvested from 10 mice per diet group and subjected to Aβ ELISA analysis as above. In addition, whole brains representing eight lithium carbonate fed PDAPP (APP_{V717F}) mice and eight base diet fed PDAPP (APP_{V717F}) mice were removed and sectioned at 10-μm coronal sections for immunocytochemistry Aβ plaque-burden analysis.

GSK3β^{S9A} Transgenic Mice. Transgenic mice were generated by pronuclear microinjection in FVB strain mice, as previously described (*24*). Human GSK3β^{S9A} was overexpresssed in the CNS of transgenic mice using a PDGF B-chain gene promoter-constitutively active GSK3β-HA-Tag transgene (*25*). Hippocampus tissue was harvested from 10, 10-month old transgenic and wild-type littermate mice and subjected to murine Aβ ELISA analysis (*26*). Additional 10-month-old GSK3β^{S9A} transgenic mice ($n = 10$) were treated with LiCl or NaCl at an oral dose of 300 mg/kg for 12 h. Upon CO_2 anesthesia, the hippocampus region was dissected out and analyzed by Aβ ELISA.

Cell Culture, Treatment, and Lysis. HEK293 $swAPP_{751}$ and $wtAPP_{751}$ stable clones were cultured in Dulbecco's Modified Eagle Medium (DMEM-F12 3:1) (Life Technologies, Gaithersburg, MD) supplemented with 5% FBS, 20 mM HEPES, 50 μg/mL tobramycin, and 300 μg/mL G418 and maintained at 37 °C and 5% CO_2. HEK293 cells were grown under similar conditions but in media lacking G418. Each cell line was plated to 50−80% confluence 1 day prior to analysis. Stable $swAPP_{751}$ HEK293 cells were treated with mood stabilizers, LiCl and VPA (Sigma) in six-well culture dishes at six different concentrations for 24 h and then harvested. At the time of the cell harvest, supernatants (media) were removed and diluted with Specimen buffer (see below under Aβ ELISA) for Aβ ELISA analysis, while cells were harvested using trypsin-EDTA and washed 1 time in PBS. The cell pellets were lysed in cell-extraction buffer (10 mM Tris at pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM NaF; 20 mM $Na_4P_2O_7$; 2 mM Na_3VO_4; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 1 mM PMSF and protease inhibitor cocktail from Sigma) for 30 min on ice with vortexing at 10-min intervals. The amount of cell-extraction buffer used was contingent upon the cell number. Cell extracts were then transferred to microcentrifuge tubes and centrifuged at 13 000 rpm for 10 min at 4 °C. Cleared lysate was transferred to a clean microfuge tube. The total protein concentration of the cell lysates was determined using the BCA protein assay kit (Pierce, Rockford, IL) according to the instructions of the manufacturer. Cell lysate was subjected to immunoblot analysis using a polyclonal anti-β-amyloid precursor protein antibody (CT695) (Zymed, San Francico, CA), derived from the C terminus of human β-amyloid precursor protein, to detect cellular APP levels, while conditioned media was subjected to immunobot analysis using the monoclonal antibody 6E10 (Signet, Dedham, MA) to detect soluble APPα (sAPPα) as previously described (*27*).

GSK3βKK DNA Construct and GSK3β Antisense Oligonucleotide. Using the polymerase chain reaction (PCR), lysine 85 and 86 of human GSK3β were changed to methionine and alanine, respectively, as described previously (*28*). GSK3βKK cDNA was cloned along with a C-terminal hemagglutinin (HA) tag into a pcDNA 3.0 (Clontech, Palo Alto, CA) expression vector at a *Nhe* I site. GSK3β phosphorothioate antisense oligonucleotide ISIS 117438 and random control oligonucleotide ISIS 129692, with sequences TGGCTTGATATACCACACCA and ACATGGGCGCGCGACTAAGT, respectively, were synthesized by ISIS Pharmaceuticals, as previously described (*29*).

Transient Tranfections. Stable $swAPP_{751}$ HEK293 cells were transiently tranfected with either an empty pcDNA 3.0 vector, the GSK3β KK kinase-deficient construct, GSK3β antisense oligonucleotide, or random control oligonucleotide from ISIS. GSK3β KK construct and vector tranfections were carried out for 24 h at 37 °C using FuGene 6 (Roche, Indianapolis, IN) at a ratio of 1 μg of DNA to 3 μL of FuGene 6, while GSK3β antisense oligo transfections were delivered using Lipotectin (Gibco, Rockville, MD) for 6, 24, and 48 h, following the standard ISIS protocol. Cell media and cell pellets were harvested and prepared as above. Media was analyzed by Aβ ELISA, while cell lysate was subjected to immunoblot analysis using a GSK3 antibody (Upstate, Lake Placid, NY) that recognizes both α and β isoforms.

Electrophoresis and Western Immunoblotting. The NuPAGE Bis-Tris System (Invitrogen Life Technologies, Carlsbad, CA) was utilized for all eletrophoresis and Western transfer using 12% precast Bis-Tris gels as previously described (*27*). All protein bands were scanned using a UMAX PowerLook 2100XL scanner.

GSK3β Kinase Activity Assay. GSK3β kinase activity was carried out using a whole-cell or whole-homogenate-kinase assay approach, as previously described (*30*).

MTT Assays. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) reagent (Sigma, St Louis, MO) was used in a colorimetric assay to determine cell viability and assess cytotoxicity. MTT is converted to

an insoluble purple formazan by active mitochondrial dehydrogenase enzymes of living cells. The insoluble formazan is solubilized using 2-propanol and subsequently measured spectrophotometrically yielding absorbance as a function of the concentration of the converted dye. Assays were performed by adding 10 mg/mL MTT directly to culture wells, incubating for 1− 4 h, and then, following removal of the medium, adding 1 mL of 0.05NHCl diluted in 2-propanol and incubating 10−20 min at room temperature. After incubation, absorbance was read at 596 nm using a BioRad model 3550 96-well microplate reader.

Aβ ELISA. Determination of total Aβ, $A\beta_{x-40}$, and $A\beta_{x-42}$ was quantified by a sandwich ELISA, as described previously (*31*). Briefly, cell samples as well as $A\beta_{x-42}$ and $A\beta_{x-40}$ standards (0−1000 pg/mL) (Bachem, Torrance, CA) were diluted in specimen buffer (0.6% BSA, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM $NaH_2PO_4 \cdot H_2O$, 145 mM NaCl, 0.05% thimerosal, and 0.05% Triton X-405), loaded onto immulon-4 microtiter plates (CMS, Chicago, IL) coated with 15 μg/mL 266 capture antibody (raised against residues 13−28 of human Aβ) for total Aβ, 15 μg/mL 2G3 (raised against residues 33−40 of human Aβ) for $A\beta_{x-40}$, and 5 μg/mL 21F12 (raised against residues 33−42 of human Aβ) for $A\beta_{x-42}$, and incubated overnight at 4 °C. Homogenate tissue samples are diluted 1:10 in ice-cold casein buffer (5.5 M guanidine-HCl and 50 mM Tris at pH 8.0). Diluted samples were added to Durapore 96-well filter plates (Millipore) and vacuum-filtered using a multiscreen filtration system vacuum manifold. Supernatants were collected in 96-well polypropylene microtiter plates and 100 μL of sample was transferred to 96-well immulon-4 microtiter plates coated with capture antibody. Plates were incubated overnight at 4 °C. The following day, plates (with cell or tissue samples) were then washed with PBS containing 0.05% Tween-20 at pH 7.4, followed by incubation for 1 h at room temperature with 3D6 biotinylated reporter antibody (Harlan, Madison, WI) specific for $A\beta_{1-5}$ (diluted 1:2000 in 0.25% casein buffer). Plates were again washed as indicated above and then incubated for 1 h at room temperature with streptavidin-horseradish peroxidase conjugate (Amersham Life Sciences, Arlington Heights, IL) diluted 1:1000 in 0.25% casein buffer. After a final series of washes, TMB-substrate (Pierce, Rockford, IL) was added for 15 min at room temperature. The enzymatic reaction was stopped with the addition of 2 N H_2SO_4. Reaction products were quantified by measuring the absorbance difference at 450 and 650 nm.

Aβ Plaque-Burden Quantitative Analysis. Coronal sections (10 μm) of PDAPP (APP_{V717F}) mouse brain from control diet and lithium carbonate fed mice were fixed at 4 °C with 4% paraformaldehyde in phosphate-buffered saline (PBS). Immunocytochemistry was carried out using a DAKO Autostainer (serial no. DC-3400-6369-02). Briefly, sections were incubated with 3D6 (Harlan) antibody for 1 h at 4 °C. After the PBS wash, sections were further incubated with Alexa Flour 488 goat anti-mouse IgG (Molecular Probes A-11029) for 60 min and mounted with a cover slip using Dako Fluorescent Mounting medium S3023 (DAKO Corporation, Carpintena, CA). Plaque pathology was captured with a Leica DMRB microscope. Quantitative evaluations were carried out on hippocampus and cortex using Image Pro Plus.

Lithium and VPA Measurement in Plasma and Brain. Measurement of lithium and VPA in the plasma and brain of PDAPP transgenic mice was carried out by ToxMed Reference Laboratory (MeHarry Medical College, Nashville, TN) using a proprietary assay method.

Statistics. All statistical analysis was completed using the Dunnett's test or student *t* test with a standard *p* value threshold of ≤ 0.05.

RESULTS

Lithium Lowers Aβ in the Brains of PDAPP (APP_{V717F}) Mice. To investigate the role of lithium in APP processing, we used the well-studied PDAPP (APP_{V717F}) transgenic mouse model of Alzheimer's disease. PDAPP (APP_{V717F}) mice overexpress the human amyloid precursor protein containing the Indiana familial AD mutation (*32*) and develop an age-dependent Aβ deposition and AD-like plaque pathology (*33*). PDAPP (APP_{V717F}) mice were treated orally with lithium (300 mg/kg) followed by the measurement of total Aβ and $A\beta_{x-42}$ peptides in the hippocampus of the animals by a sensitive Aβ ELISA. To ensure that any effect of lithium chloride on Aβ processing was not influenced by osmotic changes, sodium chloride was used as a control. As shown in Figure 1A, lithium significantly lowered both total Aβ (21%) and $A\beta_{x-42}$ (28%) in the APP_{V717F} hippocampus tissue as compared to the control group following an oral gavage for 3 h. Lithium concentration in the plasma was 0.658 mM, well within therapeutic range (0.6−1.2 mM) for the treatment of bipolar disorder, while lithium concentration in the brain was determined to be 4982.67 ng/g. There was no detectable lithium in plasma and/or brain samples of the Na^+-treated group. We next increased the lithium dose and the duration of treatment. As shown in parts C and D of Figure 1, lithium treatment at 300 mg/kg or 600 mg/kg for 12 h produced a plasma $[Li^+]$ at 1.1 and 5.9 mM, respectively, and lowered both total Aβ (21 and 31%, respectively) and $A\beta_{x-42}$ (26 and 45%, respectively) in the hippocampus of APP_{V717F} mice. These results demonstrate that lithium regulates APP processing in vivo in a suggested dose-dependent manner. Even though brain levels of lithium were not determined for the later experiment, preliminary data demonstrates that, although lithium plasma concentrations are significantly divergent depending on the dose administered, lithium brain concentrations remain fairly constant (data not shown). This would explain why we see similar, almost identical, effects on Aβ lowering, and yet there is a doubling of the plasma concentration in the 600 mg/kg dose compared to the 300 mg/kg dose (Figure 1C). VPA, another commonly used mood stabilizer with a distinct molecular structure from lithium, is also known to inhibit GSK3β, although through a divergent yet unknown and perhaps indirect mechanism. Therefore, to determine if Aβ lowering is a common effect elicited by these mood stabilizers, we orally dosed PDAPP (APP_{V717F}) mice with VPA, at 400 mg/kg for 3 and 15 h. As shown in Figure 1E, VPA also significantly lowered both total Aβ (22 and 31%, respectively) and $A\beta_{x-42}$ (28 and 33%, respectively) in the hippocampus, while VPA plasma concentration measured following 3-h gavages was in the therapeutic dose range for the treatment of bipolar disorder (0.553 mM) (Figure 1F). Plasma VPA levels at 15 h and brain VPA levels at 3 and 15 h were not determined. The effect of lithium and VPA on APP processing is strikingly similar, both

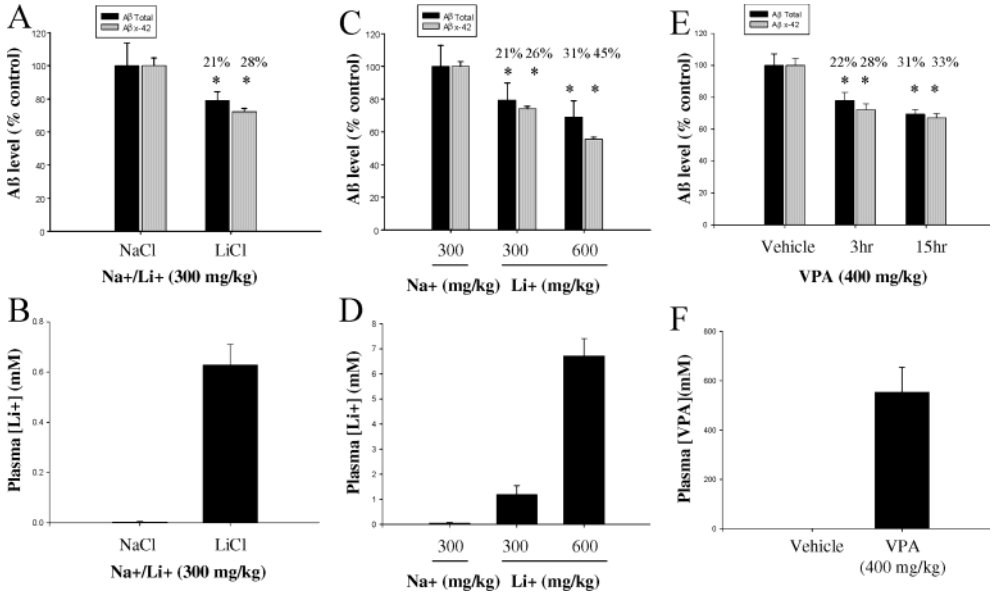

FIGURE 1: Lithium and VPA significantly reduce Aβ_{total} and Aβ_{x-42} levels in PDAPP (APP_{V717F}) mouse hippocampus as compared to the control groups. (A) PDAPP (APP_{V717F}) 3-month-old mice were administered 300 mg/kg LiCl ($n = 10$) or NaCl ($n = 10$), to control for osmotic variability, via gastric gavages. A total of 3 h following LiCl or NaCl exposure, Aβ_{total} and Aβ_{x-42} levels in the hippocampus of the mice were quantified by Aβ sandwich ELISA. Data shows that 300 mg/kg lithium orally administered for 3 h significantly lowers both total Aβ (21%) and Aβ_{x-42} (28%). The lithium concentration was measured in the plasma (B) and brain (data not shown) following the 3-h 300 mg/kg oral dose in the PDAPP (APP_{V717F}) mice depicted in A. Lithium orally dosed at 300 mg/kg showed a plasma [Li^+] of 0.685 mM, well within the therapeutic range of 0.6−1.2 mM, and a brain level of 4982.67 ng/g. (C) Additionally, 3-month-old PDAPP (APP_{V717F}) mice were further orally treated with 300 and 600 mg/kg LiCl ($n = 10$) or NaCl ($n = 10$) as a control. A total of 12 h following the treatment, Aβ_{total} and Aβ_{x-42} levels in the hippocampus of the mice were quantified by Aβ sandwich ELISA. The data demonstrates a significant dose-dependent lowering of Aβ (percent inhibition noted on chart). Lithium orally delivered to PDAPP (APP_{V717F}) mice in C produced a plasma [Li^+] of 1.1 mM at the 300 mg/kg dose (D) and 5.9 mM at the 600 mg/kg dose. Brain levels of lithium were not determined. (E) In addition, 3-month-old PDAPP (APP_{V717F}) mice were treated with VPA ($n = 10$) or water vehicle ($n = 10$) by gastric gavages at 400 mg/kg. At 3 and 15 h following the treatment, Aβ_{total} and Aβ_{x-42} levels in the hippocampus of the mice were quantified by Aβ sandwich ELISA. As revealed by the data, VPA at 400 mg/kg gastric gavages significantly reduced both total Aβ and Aβ_{x-42} (percent inhibition noted on chart). VPA plasma concentration in the PDAPP (APP_{V717F}) mice treated for 3 h in E was found to be 0.553 mM (F), again well within the therapeutic range. Plasma VPA levels at 15 h and brain levels of VPA were not determined. Error bars represent the standard deviation. The asterisks indicate a significant difference by *t* test and the Dunnet's test, $p < 0.05$.

significantly lowering in vivo total Aβ and Aβ_{x-42} to a comparable extent at exposure levels mimicking clinical relevant concentrations for the treatment of bipolar disorder. No obvious behavior abnormalities were observed in any of the mice following lithium or VPA treatment. These studies suggest that these two drugs for bipolar disorder treatment may act on a common signaling pathway that modulates APP processing in the brains of PDAPP (APP_{V717F}) transgenic mice.

Regulation of Aβ Production in Cell Cultures by Lithium and VPA. We next compared the effect of lithium and VPA on APP processing in HEK293 cells that harbor human APP_{751} containing a Swedish mutation, a well-studied cellular system that produces a significant amount of Aβ peptides, including Aβ_{x-40} and Aβ_{x-42}, in the media. The cells were treated with lithium or VPA at varying concentrations. Aβ_{total}, Aβ_{x-40}, and Aβ_{x-42} from the media were then measured and quantified by Aβ ELISA. As shown in Figure 2, both lithium and VPA significantly lowered Aβ_{total}, Aβ_{x-40}, and Aβ_{x-42} without causing significant cell death as measured by methylthiazoletetrazolium (MTT) analysis (parts A, B, D and E of Figure 2). To determine whether lithium or VPA elicit their respective Aβ lowering effects through altering cellular APP, cellular APP levels were analyzed by Western analysis using antibody CT695 (Zymed, San Fracisco, CA) derived from the C terminus of the human β-amyloid precursor protein. We found that holo-APP levels in $swAPP_{751}$ cells was not altered by lithium or VPA (parts C and F of Figure 2), suggesting an effect on APP processing or Aβ stability. In addition, the relative concentrations of total sAPPα, representing the N-terminal fragment of APP after β cleavage, in media were assessed by immunoblotting using antibody 6E10 (Signet, Dedham, MA). After lithium and VPA treatment of $swAPP_{751}$ cells, immunoblot analysis of sAPPα revealed no change (parts C and F of Figure 2). Lithium demonstrates a significant dose-dependent inhibitory effect on Aβ peptides at 3−6 mM, which is consistent with the reported effects of lithium on GSK3 signaling in several cultured cells (*34*, *35*) as well as on APP processing in COS7 cells (*36*) and τ phosphorylation in SY5Y cells (data not shown). VPA (parts D−F of Figure 2) also shows a dose-dependent inhibitory effect on APP processing, lowering Aβ_{total} (10 and 14%, respectively), Aβ_{1-40} (10 and 15%, respectively) and Aβ_{x-42} (13 and 22%, respectively) in the cell cultures with a significant effective dose at 2 mM. It is apparent that higher concentrations of lithium and VPA are required in cultured cells compared to the plasma to effectively inhibit APP processing.

Inhibition of Aβ Production by a GSK3β Kinase-Deficient Construct and Antisense Oligonucleotide. Lithium is a well-known GSK3 inhibitor in vitro (*13*, *17*) and in vivo (*14*, *15*). It is also known to inhibit the Akt/GSK3 signaling pathway by modulating Akt activity (*16*). In addition, VPA is known to inhibit GSK3 kinase (*18*). To ascertain the involvement

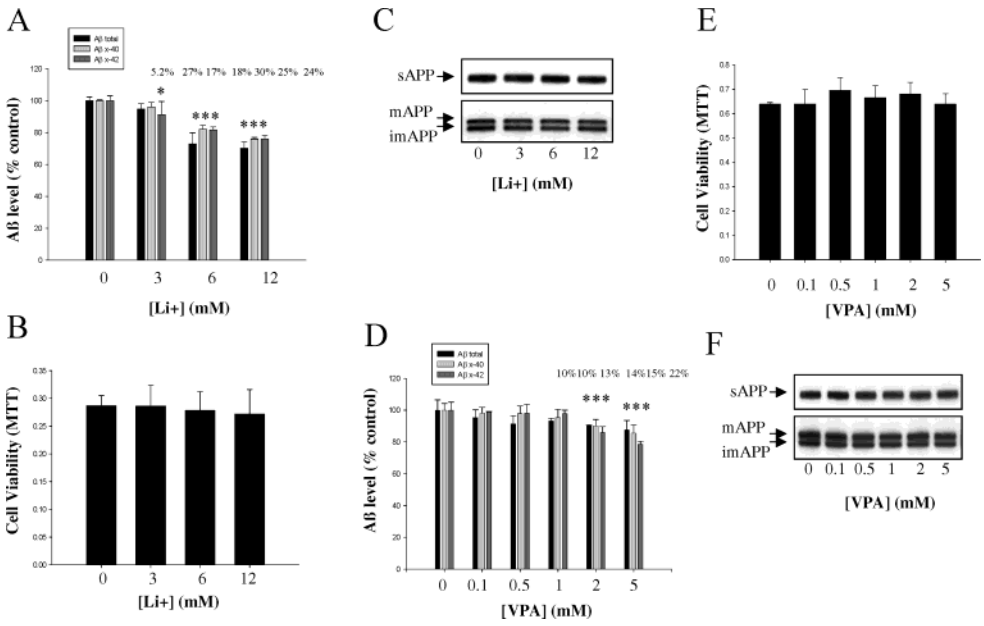

FIGURE 2: Lithium and VPA inhibit Aβ_{total}, Aβ_{x-40}, and Aβ_{x-42} production in the media of cultured cells. Subconfluent swAPP$_{751}$ HEK293 cells were treated with varying concentrations of LiCl, as indicated for 24 h. Aβ_{total}, Aβ_{x-40}, and Aβ_{x-42} in the conditioned media were quantified by Aβ sandwich ELISA. In addition, media was subjected to Western analysis using antibody 6E10, which detects sAPPα. Further, the total cell lysates were subjected to immunoblot analysis using anti-β-amyloid precursor protein C-terminal antibody (CT695) to assess APP expression. (A−C) Representative data from three independent experiments. The results show that lithium significantly lowers Aβ_{total}, Aβ_{x-40}, and Aβ_{x-42} in these cells without effecting the total or soluble APP or causing significant cell death as measured through MTT analysis. (D−F) Representative data of three independent experiments, where swAPP$_{751}$ HEK293 cells were treated with VPA and analyzed as with lithium. Like lithium, VPA also demonstrates a dose-dependent inhibitory effect on APP processing, lowering Aβ_{total}, Aβ_{x-40}, and Aβ_{x-42} in the cell cultures with a significant effective dose at 2 mM without effecting the total APP levels, sAPPα, or cell viability as measured through MTT analysis. Error bars represent the standard deviation. The asterisks indicate a significant difference by *t* test and the Dunnet's test, $p < 0.05$. mAPP, mature amyloid precursor protein; imAPP, immature amyloid precursor protein; and sAPP, soluble APPα.

of GSK3 signaling in the observed APP processing, we employed the use of a dominant negative GSK3β kinase-deficient construct and specific antisense oligonucleotide (ASO) technology, developed by the ISIS corporation, to knock down the GSK3β activity or protein. SWAPP$_{751}$ HEK293 cells were transiently transfected with a HA-tagged GSK3β kinase-deficient construct (GSK3βKK) for 24 h followed by Aβ ELISA analysis. GSK3β construct expression is readily observed by anti-HA immunoblot analysis (Figure 3A), while GSK3 activity is deficient as compared to transfected wild-type (Wt) or constitutive active (S9A) constructs (Figure 3B). GSK3βKK functional activity has been demonstrated in the stability of β catenin and E cadherin, as previously described (*30*) and represented in Figure 3C. Also shown in Figure 3, the GSK3βKK kinase-deficient construct significantly lowered Aβ_{total} and Aβ_{x-42} as compared to the vector alone (71 and 49%, respectively) or untransfected controls (Figure 3D) without affecting cell viability (Figure 3E). The ASO, however, was designed to be GSK3β-specific and in fact selectively reduced GSK3β protein expression, without affecting GSK3α protein, in the swAPP$_{751}$ cells (Figure 4A), as compared to the random negative control oligonucleotide. Knock down of the GSK3β protein was quantified by densitometry analysis as shown in Figure 4B demonstrating that GSK3β protein was reduced in a time-dependent manner, with ~60% reduction of GSK3β protein at 48 h following ASO treatment. Media representing the 48-h ASO treatment was subjected to Aβ ELISA analysis. Although the untreated control and the random negative control oligonucleotide for GSK3β ASO facilitated no response on APP processing, the GSK3β ASO significantly inhibited Aβ production, including total Aβ (31%) and Aβ_{x-42} (22%) (Figure 4D). These data are consistent with the effect of lithium or VPA on APP processing, suggesting the involvement of GSK3 in APP processing. As noted, GSK3α protein was not significantly affected by the specific GSK3β ASO (Figure 4A), and a compensatory effect of GSK3α for the loss of GSK3β could explain the only ~30% Aβ lowering effect even though GSK3β was reduced by ~60% following ASO treatment.

Lithium Blocks GSK3β-Mediated Increase in Aβ Accumulation in the Hippocampus of GSK3β Transgenic Mice While Reducing Plaque Pathology in the Brains of PDAPP (APP$_{V717F}$) Mice. We further investigated whether GSK3β regulates APP processing in vivo by overexpressing an active form of human GSK3β (GSK3β^{S9A}) tagged with HA under the control of a PDGF promoter in the mouse brain (parts A and B of Figure 5). Immunocytochemistry with the HA-tag antibody shows that human GSK3β is preferentially expressed in the neurons (Figure 5C). GSK3β activity was also moderately increased in brain tissue samples of GSK3β transgenic (parts D and E of Figure 5), consistent with previous finding that a robust increase in GSK3β activity causes lethality in the GSK3β transgenic (*24*−*26*, *28*). Next, we measured the Aβ content derived from the mouse hippocampus using a mouse Aβ ELISA and found that Aβ levels were significantly increased (19%, $p < 0.05$) in the hippocampus of 10-month-old transgenic mice as compared to the age-matched wild-type littermates. As shown in the Figure 5G, treatment of the GSK3β transgenic mice for 12

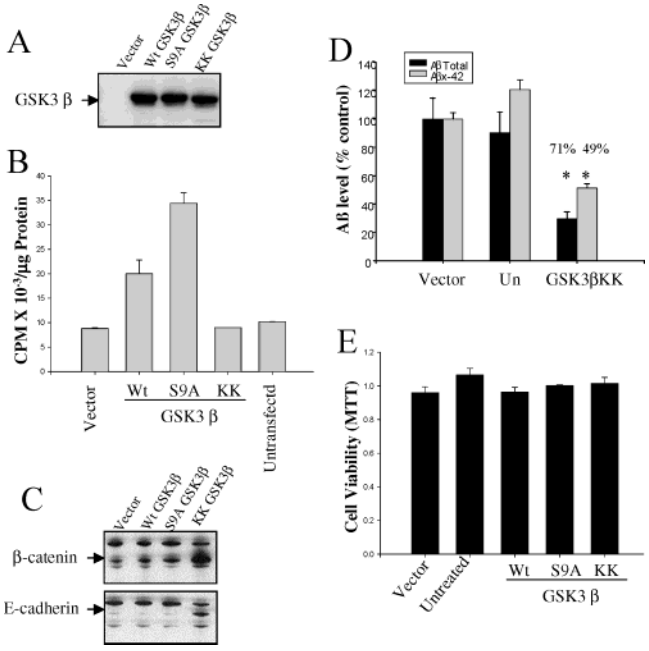

FIGURE 3: GSK3β kinase-deficient construct significantly lowers Aβ_{total} and Aβ_{x-42} as compared to the vector alone or untransfected controls in swAPP$_{751}$ HEK 293 cells. Subconfluent swAPP$_{751}$ HEK 293 cells were transiently transfected with a vector (pcDNA3.1) or a GSK3β kinase-deficient construct (GSK3βKK, tagged with HA) cDNA using FuGene 6. Overexpression of transfected GSK3β constructs are readily observed by Western blot (A) in the swAPP$_{751}$ HEK293 cells 24 h following transfection using an anti-HA specific antibody. GSK3 kinase activity (B) was also readily detected in the GSK3β transfected swAPP751 HEK293 cells following a whole-cell lysate kinase assay (Wt, wild type; S9A, constiuative active; and KK, deficient construct). (C) Representative functional GSK3βKK activity eliciting stability of β catenin and E cadherin as previously described (*30*). A total of 24 h following GSK3βKK transient transfection, Aβ_{total} and Aβ_{x-42} in the conditioned media were measured by Aβ ELISA analysis. (D) GSK3βKK significantly lowered the concentration of both Aβ_{total} (71%) and Aβ_{x-42} (49%) as compared to the vector alone or untransfected controls without effecting cellular viability (E) as measured through MTT. All data are representative of three or more independent experiments. Error bars represent the standard deviation. The asterisks indicate a significant difference by *t* test and the Dunnet's test, $p < 0.05$.

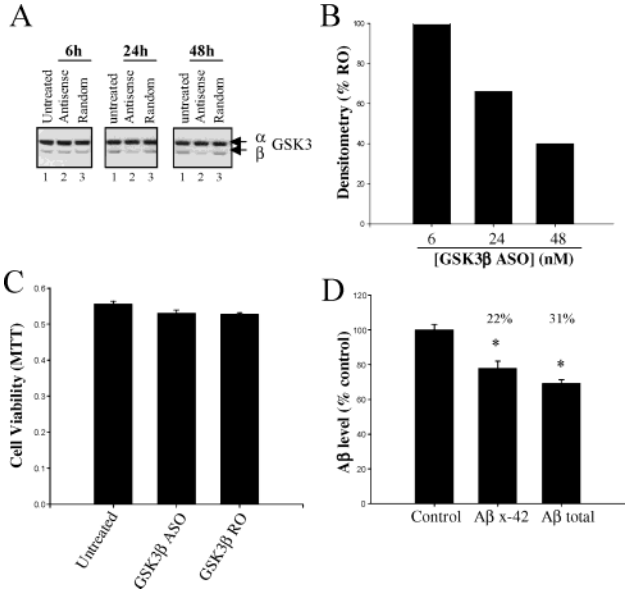

FIGURE 4: GSK3β antisense oligonucleotide (ASO) elicits select reduction of GSK3β protein expression and significantly inhibits Aβ_{total} and Aβ_{x-42} production in swAPP$_{751}$ HEK293 cell media. (A) swAPP$_{751}$ HEK293 cells were treated with GSK3β antisense (ASO, 50 nM) or random control oligonuleotide (RO, 50 nM) for 48 h followed by Western blot analysis for GSK3β and GSK3α protein expression (lane 1, untreated; lane 2, GSK3β ASO; and lane 3, random oligonucleotide). GSK3β antisense elicited a select reduction of GSK3β protein without effecting GSK3α in the swAPP$_{751}$ HEK293 cells as compared to the random negative control oligonucleotide. (B) Protein knock down of GSK3β was quantified by densitometry analysis normalized to random control oligonucleotide. GSK3β protein was reduced in a time-dependent manner with ~60% reduction of the GSK3β protein following 48 h of ASO treatment with no significant cell death as measured by MTT (C) and with no change in the GSK3α protein levels. Conditioned media were collected from the swAPP$_{751}$ HEK293 cells following GSK3β ASO treatment in A for Aβ_{total} and Aβ_{x-42} sandwich ELISA analysis. The GSK3β ASO significantly reduced the Aβ_{total} (31%) and Aβ_{x-42} (22%) production (D). Error bars represent the standard deviation. The asterisks indicate a significant difference by *t* test and the Dunnet's test, $p < 0.05$.

h with lithium (300 mg/kg) abolished the GSK3β-mediated increase in the Aβ levels, demonstrating an in vivo role for GSK3β in APP processing and the ability of lithium to attenuate the GSK3β-mediated Aβ production in vivo. Considering the moderate increase in GSK3β activity in the GSK3β transgenic mice, it is well expected that the induced level of Aβ by overexpressing GSK3β in the FVB mouse brains is also moderate, particularly when we measure the endogenous mouse Aβ level, which is notably lower as compared to the PDAPP (APP$_{V717F}$) mice.

Finally, we tested whether modulation of APP processing by lithium could ultimately lead to a reduction of plaque deposition in the PDAPP (APP$_{V717F}$) mice. Homozygous PDAPP (APP$_{V717F}$) mice show an early but age-dependent plaque pathology beginning at 3−4 months of age. Therefore, we began lithium treatment of mice at 1 month of age by introducing a food pellet diet containing lithium carbonate (*37*) and continued treatment for a period of 7 months. A group of control animals were fed a base diet of food pellets containing no lithium carbonate. After 7 months on control or lithium carbonate feed diets, lithium concentration in plasma was measured and Aβ levels in the hippocampus of the mice were quantified following Aβ sandwich ELISA as shown in Figure 6D. Consistent with the acute treatment of lithium (3−24 h), chronic treatment with a lithium carbonate diet significantly lowered Aβ levels, including Aβ_{total} and Aβ_{x-42}, in hippocampus tissues as compared to the control animals not on the lithium diet, while plasma [Li^+] was maintained at 0.9 mM (Figure 6E) and brain lithium concentration was maintained at 8000 ng/g (data not shown). No lithium could be detected in the plasma or brain in animals fed with control diets. In concert with this biochemical analysis, whole brain tissue of mice following chronic treatment was subjected to immunostaining analysis with Aβ antibody. Although Aβ immunostaining was robust in the hippocampus of mice from the control group, the Aβ deposits in the hippocampus of the lithium-treated group were significantly reduced (parts A and B of Figure 6). The Aβ deposits were subsequently quantified following image analysis using Image Pro Plus as shown in Figure 6C. Our data clearly demonstrates that lithium, while at a clinically relevant plasma concentration, not only modulates APP processing, but also significantly reduces plaques in the brains of PDAPP (APP$_{V717F}$) mice.

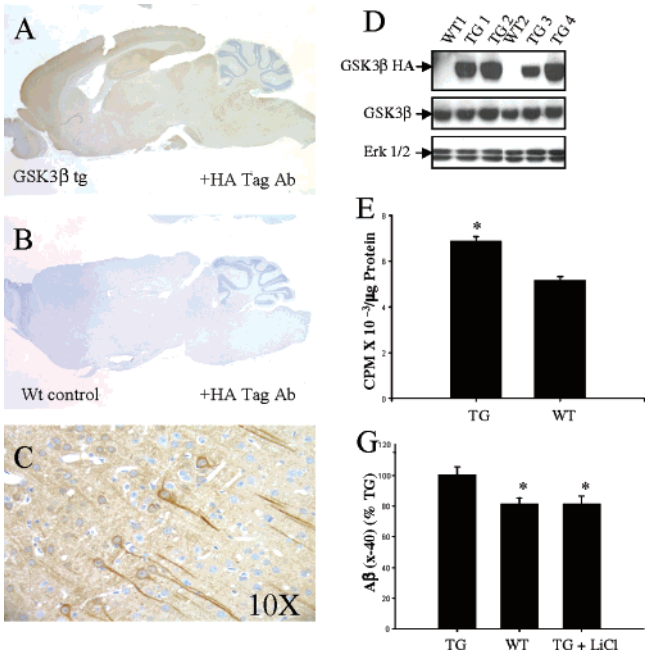

FIGURE 5: Lithium blocks GSK3β-mediated Aβ peptide accumulation in the hippocampus of GSK3β^{S9A}-HA transgenic mice. Transgenic mice expressing the human GSK3β^{S9A} transgene tagged with HA were generated by pronuclear microinjection in FVB mice under a PDGF promoter following standard methods. Brains of GSK3β^{S9A} FVB transgenic mice (A) and aged matched littermate controls (B) were prepared as sagittal sections (6 um) and immunostained with an anti-HA antibody because the human GSK3β^{S9A} transgene was tagged with HA. Strong immunostaining was detected in the brains of the GSK3β^{S9A} transgenic mice but not in the wild-type littermate controls (2.5× magnification). (C) Human GSK3β protein is highly localized in the cell body and dendrites of neurons as seen under higher magnification (10× magnification). (D) Human GSK3β^{S9A} transgene expression in the hippocampus of these mice. Immunoblotting of hippocampal homogenate with an anti-HA antibody reveals human transgene expression, while immunoblottong with an anti-GSK3β antibody alone shows moderately elevated GSK3β protein expression in the transgenic mice, as compared to the wild-type littermates. The blot was also probed with an ERK1/2 antibody to show equal protein loading. GSK3β activity (E) is elevated in the hippocampus of the GSK3β^{S9A} transgenic as measured in a whole hippocampal homogenate kinase assay using a CREB substrate peptide. Expression of the human GSK3β^{S9A} transgene significantly accumulates the Aβ levels (G) (19%) in the hippocampus as compared to the aged-matched wild-type mice (n = 10, 10-month-old GSK3β^{S9A} transgenic or wild-type littermates), while lithium treatment at 300 mg/kg for 12 h significantly lowers the GSK3β-mediated Aβ increase to wild-type levels. Error bars represent the standard deviation. The asterisks indicate a significant difference by the Dunnet's test, $p < 0.05$. WT = wild type and TG = transgenic mice.

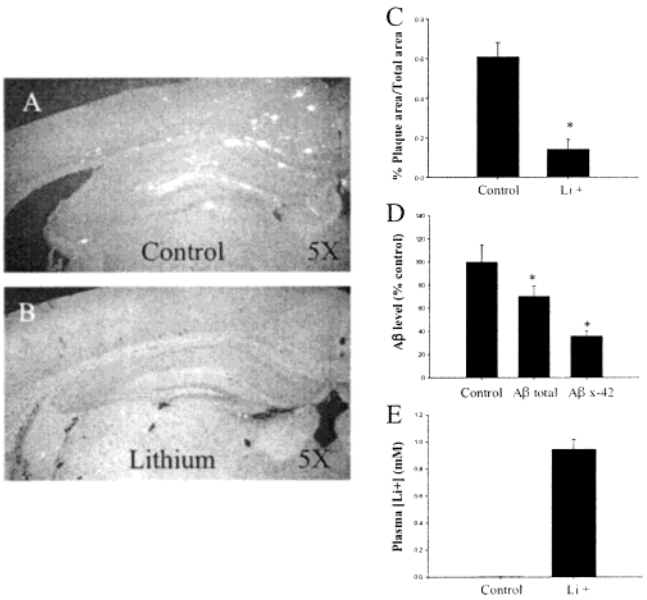

FIGURE 6: Chronic lithium treatment reduces plaque pathology in the brains of PDAPP (APP_{V717F}) mice. One-month-old PDAPP (APP_{V717F}) transgenic mice were fed either a control diet or a food pellet diet containing lithium carbonate (2.4 g/kg) for a period of 7 months and then sacrificed for Aβ analysis. After the 7 month lithium diet and upon perfusion with 1× PBS followed by fixative (4% paraformadehyde), mice brains were cut into coronal sections at 10 μm. Sections were incubated with Aβ antibody (3D6) diluents in 1:250 goat serum in PBS for 1 h. Images were captured by a Leica DMRB microscope. The plaque pathology (A and B) in the hippocampus of the lithium-diet group was significantly reduced compared to the control base-diet group. Quantitative plaque burden analysis (C) was carried out on hippocampus using Image Pro Plus providing data further showing significantly lowered plaque burden in the hippocampus. (D) $A\beta_{total}$ and $A\beta_{x-42}$ accumulations in the hippocampus of the PDAPP mice chronically treated with lithium were significantly lowered. Chronic lithium treatment (7 month) of the PDAPP transgenic mice maintained a therapeutic plasma [Li^+] of 0.9 mM (E), while brain [Li^+] was maintained at 8000 ng/g (data not shown). Error bars represent the standard deviation. The asterisks indicate a significant difference by the Dunnet's test, $p < 0.05$.

DISCUSSION

For decades, lithium has been the drug of choice for the treatment of bipolar disorder. However, the mechanism(s) of the action of lithium has not been clearly understood. Several of the prominent effects of lithium have suggested that this mood stabilizer is a selective inhibitor of GSK3β (*13*, *15*, *16*), as evidenced by mounting in vitro and in vivo studies. The negative regulation of lithium on GSK3β is likely to affect APP processing because GSK3β interacts with the known critical γ-secretase component, presenilin. Using lithium along with VPA, another commonly used mood stabilizer, as well as a GSK3β antisense oligonucleotide and GSK3β-deficient construct expression, we investigated the effect of lithium on APP processing in cells and in the brains of PDAPP (APP_{V717F}) and GSK3β transgenic mice. Our findings demonstrate that lithium regulates Aβ processing through inhibition of GSK3β, a central component in many critical intracellular signaling mechanisms.

A previous report demonstrated that lithium regulates APP processing in association with GSK3β inhibition in COS7 cells transiently transfected with APP C100, a truncated APP fragment. Although our data supports these findings, there was little direct evidence provided that this occurs in vivo and that GSK3 is directly involved in the effect (*36*). Interestingly, our observations in the HEK293 cell stably expressing $swAPP_{751}$ further compliment these data by demonstrating consistency in the need for a higher concentration of lithium above the clinically relevant dose administered for the treatment of bipolar disease to exhibit an inhibitory effect on Aβ production in mammalian cells. This apparent discrepancy in the lithium concentrations needed in vitro versus in vivo for lowering Aβ may be due to the fact that the plasma concentration may not accurately reflect lithium or VPA concentration in the brain compartment during treatment. Indeed, the brain exhibits a much higher lithium exposure, as compared to plasma, during the first

hour following lithium dosing (data not shown). In addition, preliminary data from our lab demonstrates that, although lithium plasma concentrations are significantly divergent depending on the dose administered, lithium brain concentrations remain fairly constant (data not shown). This helps explain why our in vivo lithium data show similar effects on Aβ lowering despite increased plasma levels with increasing lithium dosage (Figure 1C). Further, the stably transfected swAPP$_{751}$ cells produce large amounts of β peptides, represented predominantly by the Aβ_{40} isoform. This rapid production rate of the Aβ_{40} isoform may not be as sensitive to the inhibitory effect of lithium or VPA as the in vivo Aβ, which is produced in a much slower rate in brain tissues from PDAPP (APP$_{V717F}$) transgenic mice. When taken together, these factors could potentially contribute to the variation in the effect of lithium or VPA on APP processing observed in cells and in vivo.

The major purpose of using mutant APP is to obtain measurable Aβ levels either in the media or in the brains, because the Aβ levels, especially Aβ_{42}, are very low in the media from naive cells or the brains from wild-type mice. Clearly, the increases of the Aβ generation caused by the Swedish mutation and the Indiana mutation are through different mechanisms. While the Swedish mutation enhances BACE activity and provides more substrate for γ secretase, the Indiana mutation directly facilitates γ-secretase function. However, both models should be useful for studies of the γ-secretase-associated generation of Aβ. Our data indicates that both lithium and VPA interfere with γ-secretase activity by inhibiting GSK3, a kinase interacting directly with presenilin. Therefore, using different mutations for our in vitro and in vivo studies does not confound the explanation of our results but rather helps to clarify that the effect of lithium or VPA is not specific to the respective certain mutation.

Although APP$_{695}$ is the major isoform in the CNS, the use of the APP$_{751}$ isoform should not significantly alter our results. In fact, there has been no major difference reported between the two isoforms (APP$_{695}$ and APP$_{751}$) in terms of Aβ production in response to insults. In our understanding, GSK3 inhibitors should exert similar effects on both APP mutation isoforms. This notion is somewhat supported by our data, which show that the involvement of GSK3 in APP processing appears to be conserved in brain tissue and HEK293 cells in the presence of different mutations (Indiana or Swedish), suggesting a fundamental role of GSK3 in the process. In addition, the HEK293 cell system is well-established for APP$_{751}$ processing.

As authors were preparing this paper, Phiel et al. showed (*38*), as with our findings, that lithium regulates Aβ peptide production in Chinese hamster ovary (CHO) cells and primary mouse neurons transfected with the pathogenic APP−Swedish mutation (KM670/671NL) of APP$_{695}$. In addition, this paper showed that a daily oral dose of lithium in an AD mouse model heterozygous for the APP−Swedish transgene (Tg2576) and the PS1^{P264L} for 3 weeks elicited a reduction in Aβ production to which our acute and chronic lithium studies in the PDAPP (APP$_{V717F}$) AD mouse model concur. However, somewhat divergent from our results, this latter work suggested that Aβ production is mainly regulated by GSK3α and not GSK3β based on RNA interference studies in CHO-$_{SW}$APP$_{695}$ cells. In fact, the authors state that GSK3β may antagonize APP processing. Our results in the HEK293 cell do not support this suggestion. On the contrary, we demonstrate significant reduction of Aβ peptides following the introduction of a specific GSK3β antisense oligonucleotide or a GSK3β kinase-deficient construct suggesting a differing mechanism through GSK3 for APP processing in CHO and HEK293 cells or in APP processing associated with APP$_{695}$ and APP$_{751}$. However, GSK3α, although not specifically scrutinized in our paper, could contribute to the lithium as well as to the VPA effect that we observe because lithium and VPA are known to inhibit both GSK3α and GSK3β (*15*, *18*). One may also surmise that the GSK3β kinase-deficient construct may aberrantly affect GSK3β as well. Further, as noted in the presentation of the GSK3β antisense oligonucleotide studies, although specific knock down of GSK3β protein does trigger a reduction in Aβ production, GSK3α may have a compensatory effect as suggested when comparing the potency of the GSK3β kinase-deficient construct to the GSK3β antisense oligonucleotide in the ability to reduce Aβ peptide.

Expression of the human GSK3β transgene in our GSK3β transgenic mice elicits only a modest increase in overall GSK3 activity (~20%) and in neuronal expression of the human GSK3β transgene in the hippocampus and cortex following analysis of multiple lines. This is most likely due to the fact that increased GSK3 activity induces apoptotic cell death that may have resulted in the natural selection of a low−moderate-expressing transgenic during development. Our data is consistent with observations in previously reported GSK3β transgenics demonstrating that high expression of GSK3β is linked to lethality during the embryo development. Indeed, surviving lines usually express rather low levels of GSK3 activity (*39*−*42*). Therefore, it is not surprising but significant that we detected only a modest increase (~20%) in endogenous mouse Aβ levels in the brain tissues of the GSK3β transgenic (Figure 5). Interestingly, lithium can completely abolish the elevated Aβ level induced by the overexpression of active human GSK3β (S9A). Because of the extremely low abundance of endogenous Aβ in mouse brains, Aβ_{42} is below detectable limits. These data, coupled with our GSK3β antisense oligonucleotide results in the HEK293 cells, strongly suggest that GSK3β plays a significant role in APP processing resulting in Aβ peptide reduction in vivo. This does not, however, exclude a potentially similar effect elicited by lithium on GSK3α.

Although the mechanism by which GSK3 regulates APP processing awaits further investigation, recent studies demonstrate that GSK3β binds to presenilin 1, which is thought to constitute catalytic subunits of the γ-secretase complex in human brains and mammalian cells (*9*). Mutations in PS1 that are known to cause early onset Alzheimer's disease increase the ability of PS1 to bind to GSK3β. Therefore, it is conceivable that GSK3 regulates APP processing by deregulation of presenilin-1-dependent signaling. Indeed, lithium, a GSK3 inhibitor, has been reported to exert a positive effect on Wnt/GSK3β signaling (*43*), which is deregulated in both Alzheimer's disease and bipolar disorder. Our findings provide insights into a potential mechanism through GSK3β that underlies the clinical effect of lithium and suggest that therapeutic agents that directly or indirectly inhibit GSK3 might be effective as therapeutic interventions

in Alzheimer's disease as well as other related neurodegenerative diseases.

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