Inhibition of glycogen synthase kinase-3 β prevents sympathetic hyperinnervation in infarcted rats

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Abstract

We have demonstrated that nerve growth factor (NGF) expression in the myocardium is selectively increased during chronic stage of myocardial infarction, resulting in sympathetic hyperinnervation. Glycogen synthase kinase-3 (GSK-3) signal has been shown to play key roles in the regulation of cytoskeletal assembly during axon regeneration. We assessed whether lithium, a GSK-3 inhibitor, attenuates cardiac sympathetic reinnervation after myocardial infarction through attenuated NGF expression and Tau expression. Twenty-four hours after ligation of the anterior descending artery, male Wistar rats were randomized to either LiCl or SB216763, chemically unrelated inhibitors of GSK-3 β , a combination of LiCl and SB216763, or vehicle for four weeks. Myocardial norepinephrine levels revealed a significant elevation in vehicle-treated rats compared with sham-operated rats, consistent with excessive sympathetic reinnervation after infarction. Immunohistochemical analysis for sympathetic nerve also confirmed the change of myocardial norepinephrine. This was paralleled by a significant upregulation of NGF protein and mRNA in the vehicle-treated rats, which was reduced after administering either LiCl, SB216763, or combination. Arrhythmic scores during programmed stimulation in the vehicle-treated rats were significantly higher than those treated with GSK-3 inhibitors. Addition of SB216763 did not have additional beneficial effects compared with those seen in rats treated with LiCl alone. Furthermore, lithium treatment increased Tau1 and decreased AT8 and AT180 levels. Chronic use of lithium after infarction, resulting in attenuated sympathetic reinnervation by GSK-3 inhibition, may modify the arrhythmogenic response to programmed electrical stimulation.

Keywords: Arrhythmias, glycogen synthase kinase-3, lithium, myocardial infarction, Tau protein

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Introduction

Glycogen synthase kinase (GSK)-3 is a multifaceted protein with diverse cellular and neurophysiological functions. GSK-3 β is crucial in the regulation of nerve morphogenesis,¹ but the consequence of GSK-3 inhibition appears to be controversial. Owen and Gordon-Weeks² have shown that GSK-3 inhibition drastically prevents nerve extension. However, Fukata *et al.*³ demonstrated that GSK-3 β inhibition results in the dephosphorylation of collapsing response mediator protein 2, which leads to enhanced microtubule polymerization and axon growth. To reconcile the controversy, it has been suggested that the final outcome of GSK-3 inhibition might be determined by the extent of GSK-3 inhibition and the downstream substrates involved.⁴ Tau was reported to be a downstream target of GSK-3 β .

GSK-3 β was isolated as a kinase that was able to phosphorylate Tau.⁵ The amino terminus of Tau has been shown to be associated with the plasma membrane and can affect nerve growth factor (NGF)-induced neurite outgrowth.⁶ Thus, it is of great interest to assess whether inhibition of GSK-3 β can modulate sympathetic reinnervation by attenuated Tau-mediated NGF expression after infarction.

NGF, a member of the neurotrophin family of proteins, promotes neuronal survival and neurite outgrowth.⁷ Transmural myocardial infarction (MI) interrupts efferent sympathetic nerves and denervates viable muscle distal to MI. Levels of NGF expression within innervated tissues roughly correspond to innervation density.⁷ During sympathetic reinnervation, an important step in forming the neural circuitry is directed axon extension to reach their

synaptic targets. The cellular machinery at the nerve growth cone controls the assembly of cytoskeletal proteins and membrane components into new axons. The treatment of anti-NGF by administering antisera, target ablation, or gene disruption has been shown to prevent nerve sprouting.⁸ We have demonstrated that NGF mRNA and protein levels in the myocardium at the border zone are increased in the infarcted rats during chronic stages of MI.⁹ Increased sympathetic nerve activity plays an important role in generation of ventricular arrhythmia and sudden cardiac death.¹⁰

The effect of chronic lithium administration on nerve regeneration remained controversial. Chronic treatment with lithium increased the expression of NGF in the rat brain.¹¹ However, lithium antagonizes NGF-induced reorganization of microfilament in PC12 cells,¹² as well as NGF-induced neurite outgrowth and phosphorylation of NGF-modulated microtubule-associated proteins.¹³ The inhibitory effect of lithium on cytoskeletal rearrangement associated with cell spreading and migration is mediated by GSK-3-dependent phosphorylation.¹⁴ Lithium treatment had a strong tendency towards a reduced nerve fiber area.¹⁵ Furthermore, previous studies have shown that lithium inhibited the activity of activator protein-1.¹⁶ Deletion of the activator protein-1 element of NGF promoter markedly decreased NGF expression.¹⁷ Even though the role of lithium has previously been examined in several *in vitro* models, there are no chronic *in vivo* effects using the orally available GSK-3 inhibitor lithium on neurite branching after inducing MI over several weeks. The study aimed (1) to assess whether chronic administration of lithium chloride (LiCl) within therapeutically relevant concentrations can result in attenuated heart reinnervation through inhibition of NGF, (2) to evaluate the role of GSK-3 β and Tau phosphorylation in sympathetic innervation, and (3) to test the functional significance of attenuated heart reinnervation by ventricular pacing in a rat MI model.

Methods

The animal experiment was approved and conducted in accordance with the local ethical review committee on animal care of the China Medical University (IACUC, Permit Number: 102-63-N) and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Animals

Male Wistar rats (300–350 g) were subjected to ligation of the anterior descending artery as described previously,⁹ resulting in infarction of the left ventricular (LV) free wall. In brief, to create the model, rats were anesthetized with ketamine-xylazine (90 mg/kg and 9 mg/kg, respectively, intraperitoneally). After adequate anesthesia, they were intubated with a 14-gauge polyethylene catheter and ventilated with room air using a small animal ventilator (model 683, Harvard Apparatus, Boston, MA). The heart was exposed via a left-sided thoracotomy, and the anterior descending artery was ligated using a 5-0 silk between the pulmonary outflow tract and the left atrium. Rats were

randomly assigned so as to have approximately the same number of survivors in each group: (1) vehicle group (0.9% NaCl containing 20% dimethylsulfoxide); (2) LiCl (1 mmol/kg per day, a nonselective inhibitor of GSK-3 α/β , oral); (3) SB216763 (0.6 mg/kg every other day, 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1Hpyrrole-2,5-dione, a selective ATP competitive GSK-3 α/β antagonist, intraperitoneal injection daily in dimethylsulfoxide; Sigma, St. Louis, MO); and (4) LiCl + SB216763. The lithium dose of 1 mmol/kg per day has been shown to have plasma lithium concentrations of 0.47–0.65 mmol/L in rats, a lower therapeutic range.¹⁸ The dose of SB216763 was chosen as previous studies.¹⁹ SB216763 inhibits only GSK-3 β by 96% at 10 μ mol/L.²⁰ The drugs were started 24 h after MI, during which drugs can maximize benefits at this timing window²¹ and minimize the possibility of a direct effect on infarct size. For chronic lithium treatment, rats were given *ad libitum* water and saline to prevent hyponatremia caused by lithium-induced increased excretion of sodium. The study duration was designed to be four weeks because the majority of the myocardial remodeling process in the rat (70–80%) is complete within three weeks.²² Sham operation served as controls. In each-treated group, drugs were withdrawn about 24 h before the end of the experiments in order to eliminate their pharmacological actions.

Hemodynamics and infarct size measurements

Hemodynamic parameters were measured in anesthetized rats with ketamine-xylazine (90 mg/kg, 9 mg/kg, respectively) intraperitoneally at the end of the study. A polyethylene Millar catheter was inserted into the LV and connected to a transducer (Model SPR-407, Miller Instruments, Houston, TX) to measure LV systolic and diastolic pressure as the mean of measurements of five consecutive pressure cycles as previously described.⁹ The maximal rate of LV pressure rise (+dP/dt) and decrease (–dP/dt) was measured. After the arterial pressure measurement, the electrophysiological tests were performed. At completion of the electrophysiological tests, the atria and the right ventricle were trimmed off, and the LV was rinsed in cold physiological saline, weighed, and immediately frozen in liquid nitrogen after obtaining a coronal section of the LV for infarct size estimation. A section, taken from the equator of the LV, was fixed in 10% formalin and embedded in paraffin for determination of infarct size. Each section was stained with hematoxylin and eosin, and trichrome. The infarct size was determined as previously described.²² With respect to clinical importance, only rats with large infarction (>30%) were selected for analysis.

In vitro electrophysiological studies

To assess the potential arrhythmogenic risk of sympathetic innervation, we performed programmed electrical stimulation. To avoid the confounding effect of hormonal activation after MI on pacing-induced ventricular arrhythmias, we used the Langendorff heart as previously described.²³ Because the residual neural integrity at the infarct site is one of the determinants of the response to electrical induction of ventricular arrhythmias,²⁴ only rats with transmural

scar were included. Each heart was perfused with modified Tyrode solution (117.0 mmol/L NaCl, 23.0 mmol/L NaHCO₃, 4.6 mmol/L KCl, 0.8 mmol/L NaH₂PO₄, 1.0 mmol/L MgCl₂, 2.0 mmol/L CaCl₂ and 5.5 mmol/L glucose) equilibrated at 37°C and oxygenated with a 95% O₂/5% CO₂ gas mixture. The perfusion medium was maintained at a constant temperature of 37°C with a constant flow at 4 mL/min as described previously.²³ Atrial and ventricular epicardial ECGs were continuously recorded. After the perfusion of the isolated hearts was completed, hearts were observed for 10 min to allow stabilization of contraction and rhythm. Pacing pulses were generated from a Bloom stimulator (Fischer Imaging Corporation, Denver, CO). To induce ventricular arrhythmias, pacing was performed at a cycle length of 120 ms (S₁) for eight beats, followed by one to three extrastimuli (S₂, S₃, and S₄) at shorter coupling intervals. The endpoint of ventricular pacing was induction of ventricular tachyarrhythmia. Ventricular tachyarrhythmias including ventricular tachycardia and ventricular fibrillation were considered nonsustained when it lasted ≤15 beats and sustained when it lasted >15 beats. An arrhythmia scoring system was modified as previously described.²⁵ When multiple forms of arrhythmias occurred in one heart, the highest score was used. The experimental protocols were typically completed within 10 min.

Real-time RT-PCR of NGF

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed for samples obtained from the remote zone (>2 mm outside the infarct) as previously described.⁹ For NGF, the primers were 5'-TCCACCCACCCAGTCTTCCA-3' (sense) and 5'-GCC TTCTGCTGAGCACACA-3' (antisense). For cyclophilin, the primers were 5'-ATGGTCAACCCACCTGTTCTTCG-3' and 5'-CGTGTGAAGTCACCACCCTGACACA-3'. Cyclophilin mRNA was chosen as the internal standard because it is expressed at a relatively constant level in virtually all tissues. For quantification of NGF expression, real-time polymerase chain reaction (PCR) was performed with ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). Thermocycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 7 min. For each cDNA sample, the Ct value of the reference gene cyclophilin was subtracted from the Ct value of the sample to obtain the ΔCt. Relative expression levels were calculated. Experiments were repeated three times.

Western blot analysis of p-GSK-3β, total GSK-3β, Tau1, AT8, AT180, Tau5, NGF, and β-actin

The myocardium from the remote zone was homogenized in three volumes of Tris-buffered saline containing protease and phosphatase inhibitors (25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 30 mmol/L β-glycero-phosphate, 30 mmol/L sodium fluoride, 1 mmol/L phenyl-methylsulfonyl fluoride). The homogenates were

centrifuged and the protein concentration was determined with the bicinchoninic acid (BCA) protein assay reagent kit (Pierce). Twenty micrograms of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a nitrocellulose membrane. After incubation with antibodies, the nitrocellulose membrane was then rinsed with a blocking solution and incubated for 2 h at room temperature. Antigen-antibody complexes were detected with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride (Sigma). Films were volume-integrated within the linear range of the exposure using a scanning densitometer. Experiments were replicated three times and results were expressed as the mean value.

The following primary antibodies were used in this study: anti-p-GSK-3β (Ser9, Cell Signaling Technology), anti-total GSK-3β (BD Biosciences), anti-Tau1 (1:1000, Chemicon, CA), anti-AT8 (1:2000, Innogenetics, Belgium), anti-AT180 (1:500, Thermo Scientific), anti-Tau5 (1:2000, Calbiochem), anti-NGF (1:1000, Chemicon), and anti-β-actin (1:2000, Sigma-Aldrich). Phosphorylation-sensitive antibodies used include Tau1 (dephosphorylated residues 189-207, non-phosphorylation-dependent), AT8 (phosphorylated serine 202 and threonine 205, phosphorylation-dependent), and AT180 (phosphorylated threonine 231). We selected immunoblots for Tau1 and AT8 because previous studies have shown the great changes with these antibodies after administering neurotrophic factor.²⁶ Phosphorylation at Thr-231 is important for the ability of Tau to enhance mitogen-activated protein kinase-induced NGF response.²⁷ Besides, serine 202, threonine 205 and threonine 231 can be phosphorylated by GSK-3.²⁸ To verify that Tau phosphorylation, and not synthesis, was affected by lithium, blots were analyzed with Tau5, a phosphorylation-independent antibody that recognizes total Tau.

Immunofluorescent studies of tyrosine hydroxylase, growth-associated factor 43, and neurofilament

In order to investigate the spatial distribution and quantification of sympathetic nerve fibers, analysis of immunofluorescent staining was performed on LV muscle from the remote zone. Papillary muscles were excluded from the study because a variable sympathetic innervation has been reported.²⁹ Paraffin-embedded tissues were sectioned at a thickness of 5 μm. Tissues were incubated with anti-tyrosine hydroxylase (1:200; Chemicon), anti-growth associated protein 43 (a marker of nerve sprouting, 1:400; Chemicon), and anti-neurofilament antibodies (a marker of sympathetic nerves, 1:1000; Chemicon) in 0.5% bovine serum albumin (BSA) in PBS overnight at 37°C. The second antibody was monoclonal goat anti-mouse IgG conjugated to fluorescein isothiocyanate for tyrosine hydroxylase and rhodamine for growth-associated protein 43 and neurofilament. Isotype-identical directly conjugated antibodies served as a negative control.

The slides were coded so that the investigator was blinded to the identification of the rat sections. The nerve density was measured on the tracings by computerized

planimetry (Image Pro Plus, Media Cybernetics, Silver Spring, MD) as described previously.³⁰ The density of nerve fibers was qualitatively estimated from 10 randomly selected fields at a magnification of 400 $\times$ and expressed as the ratio of labeled nerve fiber area to total area.

Morphology and morphometry of cardiac fibrosis

Aniline blue and picrosirius staining, a collagen-specific stain (Sirius Red F3BA; Pfaltz & Bauer, Stamford, CT), were used to stain 5- μ m thick, paraffin-embedded sections at the remote area. The interstitial collagen fraction was determined by quantitative morphometry of the picrosirius-stained sections with an automated image analyzer (Image Pro Plus, CA). These parameters were assessed in a blinded fashion by at least two investigators. The densities of labeled areas were qualitatively estimated from 10 randomly selected fields at a magnification of 400 $\times$. The value was expressed as the ratio of the labeled area to total area.

Laboratory measurements

Although cardiac innervation was detected by immunofluorescent staining of tyrosine hydroxylase, growth-associated factor 43, and neurofilament, it did not imply that the nerves are functional. Thus, to examine the sympathetic nerve function, we measured LV norepinephrine levels from the remote zone. Total norepinephrine was measured using a commercial ELISA kit (Noradrenalin ELISA, IBL Immuno-Biological Laboratories Co., Hamburg, Germany).

Blood samples were collected from rats at the end of the study from the ascending aorta and serum was separated by centrifugation for the estimation of lithium levels using an Evans electroselenium (EEL)-flame photometer.

Statistical analysis

Results were presented as mean $\pm$ SD. Statistical analysis was performed using the SPSS statistical package (SPSS, version 11.0, Chicago, IL). Differences among the groups of rats were tested by a one-way analysis of variance (ANOVA). Subsequent analysis for significant differences between the two groups was performed with a multiple comparison test (Scheffe's method). Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal-Wallis test followed by a Mann-Whitney test. The significant level was assumed at value of $P < 0.05$.

Results

Differences in mortality among the infarcted groups were not found throughout the study. Relative heart weights corrected for body weight at the end of the experimental period (12 weeks of age) are presented in Table 1. Consistent with a previous study,²⁹ the gain in body weight in lithium-treated rats was less than that in the vehicle- and SB216763-treated rats despite there being no difference in weight at the start of the study. Four weeks after infarction, the infarcted area of the LV was very thin and was totally replaced by fully differentiated scar tissue. The weight of the LV inclusive of the septum remained essentially constant four weeks among the infarcted groups. The lung weight/body weight ratio, an index of lung edema, was significantly lower in the LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups compared with that in the vehicle-treated infarcted group. The values of $+dp/dt$ and $-dp/dt$ were significantly higher in the LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups compared with those in the vehicle-treated infarcted group. LV end-systolic pressure, LV end-diastolic pressure, and infarct size did not differ among the infarcted groups.

Table 1 Cardiac morphology, hemodynamics, lithium concentration, and NE concentrations at the end of study

Parameters	Sham Vehicle	Infarction treated with Vehicle	LiCl	SB216763	LiCl/SB216763
No. of rats	10	11	12	10	10
Body weight, g	412 $\pm$ 10	404 $\pm$ 10	325 $\pm$ 13*†	410 $\pm$ 17	330 $\pm$ 15*†
Heart rate, bpm	409 $\pm$ 13	421 $\pm$ 14	414 $\pm$ 13	417 $\pm$ 18	400 $\pm$ 16
LVEBP, mmHg	105 $\pm$ 5	99 $\pm$ 8	95 $\pm$ 6	98 $\pm$ 6	97 $\pm$ 9
LVEDP, mmHg	4 $\pm$ 3	18 $\pm$ 5*	15 $\pm$ 5*	14 $\pm$ 5*	15 $\pm$ 6*
$+dp/dt$, mm Hg/s	7892 $\pm$ 278	2235 $\pm$ 263*‡	2989 $\pm$ 231*	3091 $\pm$ 295*	3005 $\pm$ 283*
$-dp/dt$, mm Hg/s	7082 $\pm$ 244	2172 $\pm$ 227*‡	2844 $\pm$ 204*	2918 $\pm$ 265*	2907 $\pm$ 302*
Infarct size, %	–	41 $\pm$ 2	41 $\pm$ 2	42 $\pm$ 3	41 $\pm$ 2
LVW/tibia, mg/cm	234 $\pm$ 19	328 $\pm$ 43*	318 $\pm$ 32*	305 $\pm$ 42*	294 $\pm$ 31*
RVW/tibia, mg/cm	59 $\pm$ 11	99 $\pm$ 13*	98 $\pm$ 13*	89 $\pm$ 19*	97 $\pm$ 17*
LungW/tibia, mg/cm	378 $\pm$ 38	593 $\pm$ 53*‡	445 $\pm$ 45	459 $\pm$ 47	484 $\pm$ 39
Li, mmol/L	–	–	0.49 $\pm$ 0.15	–	0.51 $\pm$ 0.14
NE, μ g/g protein	1.06 $\pm$ 0.15	2.23 $\pm$ 0.38*‡	1.65 $\pm$ 0.27*	1.56 $\pm$ 0.24*	1.39 $\pm$ 0.25

BW: body weight; LungW: lung weight; LVEDP: left ventricular end-diastolic pressure; LVEBP: left ventricular end-systolic pressure; LVW: left ventricular weight; NE: norepinephrine; RVW: right ventricular weight. Values are mean $\pm$ SD.

* $P < 0.05$ compared with sham.

† $P < 0.05$ compared with infarcted groups treated with vehicle and SB216763.

‡ $P < 0.05$ compared with infarcted groups treated with LiCl, SB216763, and LiCl + SB216763.

Myocardial norepinephrine levels

To investigate the cardiac sympathetic function, we determined the LV norepinephrine levels. Either LiCl or SB216763 administration did not affect the basal tissue norepinephrine concentrations in the sham-operated group (data not shown). LV norepinephrine levels were significantly upregulated 2.11-fold at the remote zone in the vehicle-treated rats than in sham-operated rats (2.23 ± 0.38 vs. 1.06 ± 0.15 $\mu\text{g/g}$ protein, $P < 0.001$, Table 1). Compared with vehicle-treated rats in LiCl- or SB216763-treated rats, LV norepinephrine levels were significantly lower at the remote regions. The additional attenuated effect of SB216763 on LV norepinephrine levels was not observed compared with LiCl alone.

Immunofluorescent analyses

The tyrosine hydroxylase-immunostained nerve fibers appeared to be oriented in the longitudinal axis of adjacent myofibers (Figure 1). Tyrosine hydroxylase-positive nerve density was significantly increased in the vehicle-treated infarcted rats than that in sham. Either LiCl- or SB216763-treated rats showed significantly lower nerve density at the remote regions than vehicle-treated rats ($0.21 \pm 0.11\%$ in LiCl, $0.23 \pm 0.10\%$ in SB216763 vs. $0.39 \pm 0.15\%$ in vehicle, both $P < 0.001$, respectively). Similar to tyrosine hydroxylase results, densities of growth-associated protein 43- (Figure 1) and neurofilament-positive (data not shown) nerves were significantly attenuated in the LiCl- or SB216763-treated infarcted rats compared with those in vehicle-treated infarcted groups. The combination of LiCl and SB216763 did not further attenuate nerve density compared with either the LiCl- or SB216763-treated group. These morphometric results mirrored those of norepinephrine contents.

Myocardial fibrosis

Fibrosis of the LV from the remote area was examined in tissue sections after Sirius red staining, as shown in Figure 2. Compared with vehicle-treated infarcted rats, lithium administration showed significant attenuated fibrosis. The combination of LiCl and SB216763 did not further attenuate fibrosis compared with either the LiCl- or SB216763-treated group.

NGF protein and mRNA expression

Western blot shows that NGF levels were significantly upregulated 5.4-fold at the remote zone in the vehicle-treated infarcted rats than in sham-operated rats ($P < 0.001$, Figure 3). When compared with vehicle-treated infarcted rats, LiCl- or SB216763-treated infarcted rats had significantly lower NGF levels at the remote zone. The addition of SB216763 did not show further attenuated levels of NGF compared with LiCl alone.

PCR amplification of the cDNA revealed that the NGF mRNA levels showed a 3.3-fold upregulation at the remote zone in the vehicle-treated infarcted rats compared with sham-operated rats ($P < 0.001$, Figure 4). In LiCl- or SB216763-treated infarcted rats, the NGF mRNA levels were significantly decreased compared with those in the

vehicle-treated infarcted rats. The combination of LiCl and SB216763 showed similar NGF expression compared with either the LiCl- or SB216763-treated group.

LiCl and SB216763 reduce Tau phosphorylation

The immunoreactivities of phosphorylated and nonphosphorylated Tau were quantitated and normalized to the levels of Tau5 (total Tau), as shown in Figure 5. The ratio of anti-Tau1, which recognizes specifically dephosphorylated Tau, to anti-Tau5 increases after administering LiCl. In contrast, lithium treatment decreased AT8 and AT180. Similar results were obtained with SB216763, another structurally unrelated inhibitor of lithium. Furthermore, the additional effect of SB216763 on Tau1, AT8, and AT180 levels was not observed compared with LiCl alone. These results demonstrate that lithium and SB216763 affect the same sites of phosphorylation in Tau. Thus it is likely that lithium exerts its effects on Tau phosphorylation through the inhibition of GSK-3 β .

Electrophysiological stimulation

To further elucidate the physiological effect of attenuated sympathetic hyperinnervation, ventricular pacing was performed. Arrhythmia score in sham-operated rats was very low (0.2 ± 0.3) (Figure 6). In contrast, ventricular tachyarrhythmias consisting of ventricular tachycardia and ventricular fibrillation were inducible by programmed stimulation in vehicle-treated infarcted rats. Either lithium or SB216763 treatment significantly decreased the inducibility of ventricular tachyarrhythmias compared with vehicle. There were similar arrhythmia scores among the LiCl-, SB216763-, and the combination of LiCl and SB216763-treated infarcted rats.

Discussion

Our present study is the first one to investigate the effect of GSK-3 β inhibitors on attenuated sympathetic reinnervation after infarction. GSK-3 β signaling can regulate sympathetic innervation through Tau proteins and NGF gene expression. Furthermore, the combination of LiCl and SB216763 did not afford further protection than that afforded by each agent alone. These results support the notion that antiarrhythmic effects of LiCl is mostly, if not all, mediated by GSK-3 β inhibition. These results are consistent with the beneficial effects of GSK-3 β inhibitors, as documented structurally by a reduction in cardiac nerve sprouting, molecularly by myocardial NGF and Tau proteins, biochemically by myocardial norepinephrine, and functionally by improvement of ventricular remodeling and fatal ventricular tachyarrhythmias. Our results were consistent with previous studies, showing that lithium treatment resulted in a decrease in AT8 immunoreactivity in neuronal NT2N cells³⁰ and non-neuronal COS1 cells transiently transfected with GSK-3 and Tau.³¹ GSK-3 β inhibitors can attenuate sympathetic innervation via increased Tau1 and decreased AT8 and AT180 after infarction. Thus, as reported previously,³² GSK-3 knockin mice showed increased sympathetic activity.

Figure 1 Immunofluorescent staining for tyrosine hydroxylase and growth-associated protein 43 from the remote regions (magnification 400 $\times$). Upper, tyrosine hydroxylase. Tyrosine hydroxylase-positive nerve fibers are located between myofibrils and are oriented longitudinal direction as that of the myofibrils. Lower, growth-associated protein 43. a, sham ($n = 10$); b, infarction treated with vehicle ($n = 11$); c, infarction treated with LiCl ($n = 12$); d, infarction treated with SB216763 ($n = 10$); e, infarction treated with LiCl + SB216763 ($n = 10$). Bar = 50 μ m. Each column and bar represents mean $\pm$ SD. * $P < 0.05$ compared with sham and LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups; † $P < 0.05$ compared with sham. (A color version of this figure is available in the online journal)

The beneficial effect of lithium on sympathetic reinnervation was demonstrated by increased GSK-3 β phosphorylation and attenuated Tau phosphorylation. Our conclusions are supported by three lines of evidence.

1) Lithium and SB216763 have similar morphological and biochemical effects on sympathetic innervation in post-infarcted hearts. In the experiments described here, we have compared the effects of lithium and SB216763, two

Figure 1 Continued. (A color version of this figure is available in the online journal)

inhibitors of GSK-3 with distinct mechanisms of action. The efficacy of lithium- and SB216763-induced GSK-3 β inhibition was verified by increased phosphorylated GSK-3 β levels. Either lithium or SB216763 administration has been shown to be associated with attenuated sympathetic reinnervation after infarction with similar potency. Furthermore, addition of SB216763 attenuated sympathetic

reinnervation and did not have additional beneficial effects compared with rats treated with either LiCl or SB216763 alone, confirming the critical role of GSK-3 β in sympathetic reinnervation. Thus, this finding suggests that the morphological effects of lithium on sympathetic innervation are due to inhibition of GSK-3. The results were consistent with the findings of Kim *et al.*,³³ showing that GSK-3 has

Figure 2 Representative sections from the remote area with Sirius Red staining (red, magnification 400 $\times$) at four weeks after infarction. The line length corresponds to 50 μ m. a, sham ($n = 10$); b, infarction treated with vehicle ($n = 11$); c, infarction treated with LiCl ($n = 12$); d, infarction treated with SB216763 ($n = 10$); e, infarction treated with LiCl + SB216763 ($n = 10$). Bar = 50 μ m. Each column and bar represents mean $\pm$ SD. * $P < 0.05$ compared with sham and LiCl $^{-}$, SB216763 $^{-}$, and LiCl + SB216763-treated infarcted groups; † $P < 0.05$ compared with sham. (A color version of this figure is available in the online journal.)

Figure 3 Western blot analysis of NGF (MW: 13 kDa) in homogenates of the LV from the remote zone. When compared with vehicle-treated infarcted rats, LiCl-, SB216763-, and LiCl + SB216763-treated infarcted rats had significantly lower NGF levels by quantitative analysis. Relative abundance was obtained by normalizing the density of NGF protein against that of β-actin. Results are mean ± SD of three independent experiments. * $P < 0.05$ compared with sham and LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups; † $P < 0.05$ compared with sham

Figure 4 Left ventricular NGF mRNA levels. Each mRNA was corrected for an mRNA level of cyclophilin. Each column and bar represents mean ± SD. * $P < 0.05$ compared with sham and LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups; † $P < 0.05$ compared with sham

Figure 5 GSK-3 β inhibition is an important regulator of Tau phosphorylation. Western blot analysis in homogenates of the LV from the remote zone. When compared with vehicle-treated infarcted rats, LiCl-, SB216763-, and LiCl + SB216763-treated infarcted rats had significantly higher p-GSK-3 β and Tau1 levels and lower AT8 and AT180 levels by quantitative analysis. Relative abundance was obtained against that of β -actin. Results are mean $\pm$ SD of three independent experiments. Sham ($n = 10$); vehicle ($n = 11$); LiCl ($n = 12$); SB216763 ($n = 10$); LiCl + SB216763 ($n = 10$). * $P < 0.05$ compared with sham and LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups; † $P < 0.05$ compared with LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups

Figure 6 Inducibility quotient of ventricular arrhythmias by programmed electrical stimulation four weeks after MI in an *in vitro* model. * $P < 0.05$ compared with sham and LiCl-, SB216763-, and LiCl + SB216763-treated infarcted groups; $^{\dagger}P < 0.05$ compared with sham

been shown to be a key signaling molecule that controls axonal assembly at the growth cone via regulation of multiple microtubule-binding proteins. Besides, chronic inhibition of GSK-3 by lithium has been postulated to induce neurological toxicity by exacerbating neuronal apoptosis in mice at a serum lithium level of above 1.0 mmol/L,³⁴ a dose much higher than that used in this study. Given that progressive toxicity to marked neurological impairment correlates with increasing serum lithium levels,³⁵ the suppression of sympathetic innervation by either lithium or SB216763 in this study was not due to toxic effects because we used the same dose of lithium and SB216763 as previous studies which did not produce toxic effect and was not detrimental to nerve.¹⁹

2) Our data indicate GSK-3-induced Tau phosphorylation is associated with sympathetic reinnervation. GSK-3 is emerging as a major regulatory molecule in the growth cone for controlling local axon assembly. Axon extension is the continuous addition microtubules at the nerve growth cone. Many of these microtubule proteins such as Tau are validated GSK-3 substrates. Tau has been shown to play a role in neurite length and neurite initiation.²⁷ Tau phosphorylation influences the positioning of Tau in dendrites, and the association of Tau with plasma membranes and nuclei. Previous studies have shown that phosphorylation of Tau at Thr-231 is required to potentiate activation of activator protein-1 transcription factors, an element of NGF promoter, through the mitogen-activated protein kinase pathway.²⁷ Because GSK-3 β is typically inactivated in response to NGF signaling,³⁶ the phosphorylation of Tau at Thr-231 leads to a positive feedback mechanism that would enhance signaling. Thus, in addition to the attenuated dephosphorylated Tau-induced NGF levels, NGF can influence Tau phosphorylation through GSK-3 β inhibition.

Two proline-directed kinases, GSK-3 and cyclin-dependent kinase-5, are thought to be key factors in abnormal Tau phosphorylation.^{37,38} Because LiCl is active for the two kinases and is not completely specific for GSK-3, a second GSK-3 inhibitor, SB216763, was also tested. Treatment

correlated with reduced phosphorylation of Tau at sites known to be phosphorylated by GSK-3 β , suggesting that the inhibitors can reduce Tau phosphorylation either directly or indirectly in postinfarcted rats. Genetic evidence also supports a central role for Tau in maintaining microtubule populations in growing axons. Neurons cultured from the Tau knockout³⁹ show reduced axonal degeneration similarly after GSK-3 inhibition. Furthermore, given the similar amount of total Tau assessed by Tau5 after administering GSK-3 β inhibitors, the increased dephosphorylated Tau levels were not affected by increased degradation.

3) The severity of pacing-induced fatal arrhythmias was associated with the degree of sympathetic reinnervation. This model has the advantage of isolated preparations, with the absence of influence from circulating hormones and hemodynamic reflexes. The finding was further supported by Cao *et al.*,¹⁰ showing that increased postinjury sympathetic nerve density may be responsible for the occurrence of ventricular arrhythmia and sudden cardiac death in animals and patients.

Previous studies

Previous studies have suggested that lithium may be involved in the process of neurite growth; however, the results were not always concordant. Recently, Wang *et al.*⁴⁰ have reported the effects of lithium on neurite branching using N2a cells. Interestingly, contrary to our finding, they found that expression of lithium increased neurite branching. The basis for this apparent contradiction remains unclear. The effects of lithium on NGF-induced neurite branching were stimulating and inhibiting, depending on the types of cells or neurites.⁴⁰ Different cells may vary in their dependence on the function of various GSK-3 β substrates, such as glycogen synthase, eIF2B, Tau, and β -catenin.⁴¹ Even in the same neurons, the same neurotrophin accelerates or slows growth cones depending on additional conditions.⁴² Furthermore, GSK-3 activity must be

tightly regulated to maintain nerve integrity. A certain level of GSK-3 activity is essential for a neurite to extend as an axon, and consequently the formation of the axon initial segment.⁴³ In contrast, too low or high levels of GSK-3 activity may attenuate axonal elongation.⁴⁴ It is not surprising to know that marked inhibition of GSK-3 activity by administering GSK-3 inhibitors attenuated axonal elongation.

Similarly to the complex effect of lithium on neurite growth, there were controversies regarding the effect of lithium on cardiac fibrosis. We showed that there was significantly attenuated cardiac fibrosis in infarcted groups treated with LiCl, SB216763, and the combination of LiCl and SB216763 compared with vehicle. This result contrasted with the findings of Lal *et al.*,⁴⁵ showing an increase in the fibrotic response in cardiac fibroblast-conditioned GSK-3 β knockout mice after MI. This discrepancy can be explained at least in part, by different affected cells, different levels of GSK-3 inhibition, and different GSK-3 isoforms. Conditional deletion of GSK-3 β , specifically in cardiomyocytes, has been shown to have no effect on cardiac fibrosis after MI.⁴⁶ Besides, lithium, a nonselective GSK-3 inhibitor, inhibits both isoforms of GSK-3. Upregulation of GSK-3 α in cardiac-specific GSK-3 α transgenic mice increases cardiac fibrosis pressure overload-induced cardiac hypertrophy.⁴⁷ It is not surprising to know that marked inhibition of GSK-3 α activity by administering lithium attenuated fibrosis. Indeed, our results were consistent with the findings of Takeda *et al.*,⁴⁸ showing that deletion of Kruppel-like factor 5 leading to GSK-3 inhibition was protective with less fibrosis in a model of thoracic aortic constriction.

Clinical implications

GSK-3 inhibitors are currently being investigated as potential therapeutics for a variety of chronic diseases such as diabetes and neurodegenerative disorders. Given that there is a high incidence of coronary artery disease in patients with stroke, many important issues require clarification before lithium can be considered to be used as an adjunctive treatment after stroke. Great care should be taken when using lithium in patients with an enhanced cardiovascular risk profile. The new effect of lithium revealed by the present study may indicate a pivotal role for lithium in inhibiting arrhythmia by attenuated sympathetic hyperinnervation. Clinical practice guidelines have long recommended lithium as a first-line long-term treatment for bipolar disorder but its use has decreased, partly because of safety concerns. Evidence confirming its efficacy has led to suggestions that lithium should be more widely used.

The present study explored the possibility that lithium might have clinical efficacy for the treatment after MI. However, several conditions may limit its clinical application. First, the clinical effects of lithium require chronic administration, with a lag period for onset of action of several days to weeks. With a few exceptions, however, most of the *in vitro* studies of their action only examined the short-term effect of these drugs. Second, the accepted plasma therapeutic ranges of lithium were between 0.6 and 1.2 mmol/L.⁴⁴ Lithium levels within the therapeutic ranges cause (at physiological levels of Mg²⁺) only an

approximately 25% inhibition of total GSK-3 activity (i.e. approximately equal to deletion of 1 allele of GSK-3 β).⁴⁹ Given the inhibitory effect of all GSK-3 inhibitors on axon elongation is dose-dependent,⁴² the effects produced by subpharmacological dose of lithium in this study cannot necessarily be extrapolated to clinical conditions.

Study limitations

The limitation of the current study is that all studies were obtained by pharmacological inhibition. A potential problem with the present study is the use of lithium as an antagonist of GSK-3 β when there are many potential nonspecific targets of lithium.⁵⁰ For example, lithium appears to inhibit mitogen-activated protein kinase-extracellular-signal-regulated kinase (MEK-ERK) signaling, a pathway regulating neuronal function, synaptic plasticity, and survival.⁵¹ Furthermore, others kinases except GSK-3 can also phosphorylate serine/threonine-directed Tau kinases such as protein kinase A, casein kinase 1, and cyclin-dependent kinase-5.⁵² A number of small molecule inhibitors of GSK-3 have now been synthesized and these provide an independent means to examine GSK-3 function.⁵³ SB216763 can only inhibit GSK-3 and failed to alter MEK-ERK phosphorylation and other kinases. Our results showed similar effects of lithium and SB216763 on sympathetic innervation. This illustrates that lithium and SB216763 in sympathetic innervation may be mediated by a common mechanism, GSK-3 inhibition.

SB216763 as a selective ATP competitive antagonist of GSK-3 β also has off-target effects. Such ATP competitive inhibitors demonstrate limited specificity, as the ATP-binding pocket is highly conserved among protein kinases. Inhibition of GSK-3 by SB216763 has been described to reduce the inflammatory response upon the activation of Toll like-receptor 2 (TLR2) signaling.⁵⁴ Interestingly, inhibition of TLR2 signaling contributes to reduced NGF receptor expression.⁵⁵ Thus, given the inhibitory effects of SB216763 on TLR2 signaling, the effects of SB216763 on TLR2 may play a role in attenuated NGF effects. However, lithium, another inhibitor of GSK-3 not closely related structurally to SB216763 that has been reported to alter TLR2 signaling only at high concentration of more than 5 mmol/L,⁵⁶ far higher than those in this study, attenuated NGF expression to a similar degree to SB216763. It is GSK-3 that might play a crucial role in NGF expression. It will be important to test this hypothesis further in future studies, in particular the identification and characterization of drug-resistant GSK-3 β mutants will provide a powerful approach in dissecting the role of GSK-3 β in sympathetic innervation. In addition, analysing the effects these inhibitors have on GSK-3 β null cells will help delineate off-target effects.

Most studies of the function of GSK-3 in neuronal morphology and physiology have focused on the more abundant GSK-3 β , although some studies have highlighted a role for GSK-3 α in axon formation.⁴³ Thus, we cannot exclude the possibility that axon formation might depend on both isoforms; particularly since the GSK-3 inhibitors used are unable to differentiate between the two of them.

Conclusions

These data show excessive myocardial reinnervation after infarction, which is modulated by GSK-3 β inhibition. Lithium administration after infarction can reduce the inducibility of ventricular arrhythmias as a result of attenuated sympathetic reinnervation probably through a GSK-3 β inhibition-dependent mechanism. This may be a new beneficial role of lithium in decreasing cardiovascular mortality. Therefore, lithium is a candidate for use in clinical trials of new therapies for MI victims.

Author contributions: T-ML conceived and designed the study, analyzed and interpreted the data, and drafted and approved the final version of the paper. S-ZL conceived the study, interpreted the data, and revised and approved the final version of the paper. N-CC conceived and designed the study, and revised and approved the final version of the paper.

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REFERENCES

- Jiang H, Guo W, Liang X, Rao Y. Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3 β and its upstream regulators. *Cell* 2005;**120**:123–35
- Owen R, Gordon-Weeks PR. Inhibition of glycogen synthase kinase 3 β in sensory neurons in culture alters filopodia dynamics and microtubule distribution in growth cones. *Mol Cell Neurosci* 2003;**23**:626–37
- Fukata Y, Itoh TJ, Kimura T, Ménager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K. CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol* 2002;**4**:583–91
- Kim WY, Zhou FQ, Zhou J, Yokota Y, Wang YM, Yoshimura T, Kaibuchi K, Woodgett JR, Anton ES, Snider WD. Essential roles for GSK-3 α and GSK-3 β in neurotrophin-induced and hippocampal axon growth. *Neuron* 2006;**52**:981–96
- Takahashi M, Tomizawa K, Kato R, Sato K, Uchida T, Fujita SC, Imahori K. Localization and developmental changes of tau protein kinase I/glycogen synthase kinase-3 β in rat brain. *J Neurochem* 1994;**63**:245–55
- Brandt R, Léger J, Lee G. Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. *J Cell Biol* 1997;**131**:1327–40
- Lockhart ST, Turrigiano GG, Birren SJ. Nerve growth factor modulates synaptic transmission between sympathetic neurons and cardiac myocytes. *J Neurosci* 1997;**17**:9573–82
- Gloster A, Diamond J. Sympathetic nerves in adult rats regenerate normally and restore pilomotor function during an anti-NGF treatment that prevents their collateral sprouting. *J Comp Neurol* 1992;**326**:363–74
- Lee TM, Lin SZ, Chang NC. Both PKA and Epac pathways mediate N-acetylcysteine-induced Connexin43 preservation in rats with myocardial infarction. *PLoS One* 2013;**8**:e71878
- Cao JM, Fishbein MC, Han JB, Lai WW, Lai AC, Wu TJ, Czer L, Wolf PL, Denton TA, Shintaku IP, Chen PS, Chen LS. Relationship between regional cardiac hyperinnervation and ventricular arrhythmias. *Circulation* 2000;**101**:1960–9
- Angelucci F, Aloe L, Jiménez-Vasquez P, Mathé AA. Lithium treatment alters brain concentrations of nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor in a rat model of depression. *Int J Neuropsychopharmacol* 2003;**6**:225–31
- Paves H, Neuman T, Metsis M, Saarma M. Nerve growth factor-induced rapid reorganization of microfilaments in PC12 cells: possible roles of different second messenger systems. *Exp Cell Res* 1990;**186**:218–26
- Burstein DE, Seeley PJ, Greene LA. Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule-associated proteins. *J Cell Biol* 1985;**101**:862–70
- Cai X, Li M, Vrana J, Schaller MD. Glycogen synthase kinase 3- and extracellular signal-regulated kinase-dependent phosphorylation of paxillin regulates cytoskeletal rearrangement. *Mol Cell Biol* 2006;**26**:2857–68
- Licht RW, Smith D, Braendgaard H. The effect of chronic lithium treatment on the calibre of axons and nerve fibres in the rat sural nerve. *Eur Neuropsychopharmacol* 1997;**7**:95–8
- Hongisto V, Smeds N, Brecht S, Herdegen T, Courtney MJ, Coffey ET. Lithium blocks the c-Jun stress response and protects neurons via its action on glycogen synthase kinase 3. *Mol Cell Biol* 2003;**23**:6027–36
- Ieda M, Fukuda K, Hisaka Y, Kimura K, Kawaguchi H, Fujita J, Shimoda K, Takeshita E, Okano H, Kurihara Y, Kurihara H, Ishida J, Fukamizu A, Fedoroff HJ, Ogawa S. Endothelin-1 regulates cardiac sympathetic innervation in the rodent heart by controlling nerve growth factor expression. *J Clin Invest* 2004;**113**:876–84
- Xu J, Scholz A, Rösch N, Blume A, Unger T, Kreutz R, Culman J, Gohlke P. Low-dose lithium combined with captopril prevents stroke and improves survival in salt-loaded, stroke-prone spontaneously hypertensive rats. *J Hypertens* 2005;**23**:2277–85
- Bergmann C, Akhmetshina A, Dees C, Palumbo K, Zerr P, Beyer C, Zwerina J, Distler O, Schett G, Distler JH. Inhibition of glycogen synthase kinase 3 β induces dermal fibrosis by activation of the canonical Wnt pathway. *Ann Rheum Dis* 2011;**70**:2191–8
- Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Brown MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD, Holder JC. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* 2000;**7**:793–803
- Xia QG, Chung O, Spitznagel H, Illner S, Jänichen G, Rossius B, Gohlke P, Unger T. Significance of timing of angiotensin AT1 receptor blockade in rats with myocardial infarction-induced heart failure. *Cardiovasc Res* 2001;**49**:110–7
- Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. *Circulation* 1990;**81**:161–72
- Lee TM, Chen CC, Chang NC. Cardiac sympathetic hyperinnervation in deoxycorticosterone acetate-salt hypertensive rats. *Clin Sci (Lond)* 2012;**123**:445–57
- Herre JM, Wetstein L, Lin YL, Mills AS, Dae M, Thames MD. Effect of transmural versus nontransmural myocardial infarction on inducibility of ventricular arrhythmias during sympathetic stimulation in dogs. *J Am Coll Cardiol* 1988;**11**:414–21
- Bélitchard P, Savard P, Cardinal R, Nadeau R, Gosselin H, Paradis P, Rouleau JL. Markedly different effects on ventricular remodeling result in a decrease in inducibility of ventricular arrhythmias. *J Am Coll Cardiol* 1994;**23**:505–13
- Elliott E, Atlas R, Lange A, Ginzburg I. Brain-derived neurotrophic factor induces a rapid dephosphorylation of tau protein through a PI-3 Kinase signalling mechanism. *Eur J Neurosci* 2005;**22**:1081–9
- Leugers CJ, Lee G. Tau potentiates nerve growth factor-induced mitogen-activated protein kinase signaling and neurite initiation without a requirement for microtubule binding. *J Biol Chem* 2010;**285**:19125–34
- Hanger DP, Noble W. Functional implications of glycogen synthase kinase-3-mediated tau phosphorylation. *Int J Alzheimers Dis* 2011;**2011**:352805
- Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev* 2001;**15**:2203–8
- Hong M, Chen DC, Klein PS, Lee VM. Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. *J Biol Chem* 1997;**272**:25326–32

31. Stambolic V, Ruel L, Woodgett JR. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr Biol* 1996;**6**:1664–8
32. Siraskar B, Völkl J, Ahmed MS, Hierlmeier M, Gu S, Schmid E, Leibrock C, Föller M, Lang UE, Lang F. Enhanced catecholamine release in mice expressing PKB/SGK-resistant GSK3. *Pflugers Arch* 2011;**462**:811–9
33. Kim YT, Hur EM, Snider WD, Zhou FQ. Role of GSK3 signaling in neuronal morphogenesis. *Front Mol Neurosci* 2011;**4**:48
34. Gomez-Sintes R, Lucas JJ. NFAT/Fas signaling mediates the neuronal apoptosis and motor side effects of GSK-3 inhibition in a mouse model of lithium therapy. *J Clin Invest* 2010;**120**:2432–45
35. Macritchie KAN, Young AH. Adverse syndromes associated with lithium. In: Haddad P, Dursun S, Deakin B (eds). *Adverse syndromes and psychiatric drugs: a clinical guide*. New York, NY: Oxford University Press, 2004, pp. 89–109
36. Kleijn M, Welsh GI, Scheper GC, Voorma HO, Proud CG, Thomas AA. Nerve and epidermal growth factor induce protein synthesis and eIF2B activation in PC12 cells. *J Biol Chem* 1998;**273**:5536–41
37. Cruz JC, Tsai LH. A Jekyll and Hyde kinase: roles for Cdk5 in brain development and disease. *Curr Opin Neurobiol* 2004;**14**:390–4
38. Sato S, Tatebayashi Y, Akagi T, Chui DH, Murayama M, Miyasaka T, Planel E, Tanemura K, Sun X, Hashikawa T, Yoshioka K, Ishiguro K, Takashima A. Aberrant tau phosphorylation by glycogen synthase kinase-3 β and JNK3 induces oligomeric tau fibrils in COS-7 cells. *J Biol Chem* 2002;**277**:42060–5
39. Dawson HN, Cantillana V, Jansen M, Wang H, Vitek MP, Wilcock DM, Lynch JR, Laskowitz DT. Loss of tau elicits axonal degeneration in a mouse model of Alzheimer's disease. *Neuroscience* 2010;**169**:516–31
40. Wang Z, Wang J, Li J, Wang X, Yao Y, Zhang X, Li C, Cheng Y, Ding G, Liu L, Ding Z. MEK/ERKs signaling is essential for lithium-induced neurite outgrowth in N2a cells. *Int J Dev Neurosci* 2011;**29**:415–22
41. Welsh GI, Wilson C, Proud CG. GSK3: a SHAGGY frog story. *Trends Cell Biol* 1996;**6**:274–9
42. Song HJ, Poo MM. Signal transduction underlying growth cone guidance by diffusible factors. *Curr Opin Neurobiol* 1999;**9**:355–63
43. Garrido JJ, Simon D, Varea O, Wandosell F. GSK3 α and GSK3 β are necessary for axon formation. *FEBS Lett* 2007;**581**:1579–86
44. Bowden CL. Dosing strategies and time course of response to antimanic drugs. *J Clin Psychiatry* 1996;**57**:4–9
45. Lal H, Ahmad F, Zhou J, Yu JE, Vagnozzi RJ, Guo Y, Yu D, Tsai EJ, Woodgett J, Gao E, Force T. Cardiac fibroblast glycogen synthase kinase-3 β regulates ventricular remodeling and dysfunction in ischemic heart. *Circulation* 2014;**130**:419–30
46. Woulfe KC, Gao E, Lal H, Harris D, Fan Q, Vagnozzi R, DeCaul M, Shang X, Patel S, Woodgett JR, Force T, Zhou J. Glycogen synthase kinase-3 β regulates post-myocardial infarction remodeling and stress-induced cardiomyocyte proliferation in vivo. *Circ Res* 2010;**106**:1635–45
47. Zhai P, Gao S, Holle E, Yu X, Yatani A, Wagner T, Sadoshima J. Glycogen synthase kinase-3 α reduces cardiac growth and pressure overload-induced cardiac hypertrophy by inhibition of extracellular signal-regulated kinases. *J Biol Chem* 2007;**282**:33181–91
48. Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, Shindo T, Sano M, Otsu K, Snider P, Conway SJ, Nagai R. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. *J Clin Invest* 2010;**120**:254–65
49. O'Brien WT, Harper AD, Jové F, Woodgett JR, Maretto S, Piccolo S, Klein PS. Glycogen synthase kinase-3 β haploinsufficiency mimics the behavioral and molecular effects of lithium. *J Neurosci* 2004;**24**:6791–8
50. Phiel CJ, Klein PS. Molecular targets of lithium action. *Annu Rev Pharmacol Toxicol* 2001;**41**:789–813
51. Grewal SS, York RD, Stork PJS. Extracellular-signal regulated kinase signalling in neurons. *Curr Opin Neurobiol* 1999;**9**:544–53
52. Hanger DP, Anderton BH, Noble W. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends Mol Med* 2009;**15**:112–9
53. Cross DA, Culbert AA, Chalmers KA, Facci L, Skaper SD, Reith AD. Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurones from death. *J Neurochem* 2001;**77**:94–102
54. Hoarau C, Martin L, Faugaret D, Baron C, Dauba A, Aubert-Jacquín C, Velge-Roussel F, Lebranchu Y. Supernatant from bifi do bacterium differentially modulates transduction signaling pathways for biological functions of human dendritic cells. *PLoS ONE* 2008;**3**:e2753
55. Wirz SA, Tobias PS, Ulevitch RJ, Aribibe L, Bartfai T. TLR2 is required for the altered transcription of p75NGF receptors in gram positive infection. *Neurochem Res* 2006;**31**:297–301
56. Ballanger E, Tenaud I, Volteau C, Khammari A, Dréno B. Anti-inflammatory effects of lithium gluconate on keratinocytes: a possible explanation for efficiency in seborrheic dermatitis. *Arch Dermatol Res* 2008;**300**:215–23

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