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BRIEF REPORT

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Lentivirally mediated GSK-3β silencing in the hippocampal dentate gyrus induces antidepressant-like effects in stressed mice

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Abstract

Inhibition of glycogen synthase kinase-3 (GSK-3) by pharmacological tools can produce antidepressant-like effects in rodents. However, the GSK-3 isoform(s) and brain region(s) involved in regulating these behavioural effects remain elusive. We studied the effects of bilateral intra-hippocampal injections of lentivirus-expressing short-hairpin (sh)RNA targeting GSK-3β on behavioural performance in mice subjected to chronic stress. Pre-injection of lentivirus-expressing GSK-3β shRNA into the hippocampal dentate gyrus significantly decreased immobility time in both forced swim and tail suspension tests, while the locomotor activity of these mice was unchanged. These results suggest that lentiviral GSK-3β shRNA injection induces antidepressant-like effects in chronically stressed mice. Under these conditions, the expression levels of GSK-3β were persistently and markedly reduced in the hippocampus following GSK-3β shRNA injection. To our knowledge, this is the first demonstration that a single injection of lentivirus-expressing GSK-3β shRNA in the hippocampal dentate gyrus of chronically stressed mice has antidepressant-like effects elicited by gene silencing.

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Key words: Antidepressant-like effects, forced swim test, GSK-3 shRNA, hippocampal dentate gyrus, lentivirus.

Introduction

Glycogen synthase kinase-3 (GSK-3) has emerged as an important target for lithium, a major mood stabilizer used to treat bipolar disorder. The α and β isoforms of GSK-3 regulate a large number of proteins through phosphorylation mechanisms, and affect pathways involved in neuroplasticity, neurotrophicity, cell survival, and neurotransmission. Lithium appears to inhibit GSK-3 activity directly by competitive inhibition of Mg^{2+} binding to the active site of the enzyme, and indirectly by enhancing serine phosphorylation levels through multiple signalling pathways including Akt (reviewed in Jope, 2003; Rowe *et al*. 2007). Other compounds used to treat bipolar disorder, such as some antipsychotic medications and the mood stabilizers valproate and lamotrigine, also enhance GSK-3β serine-9 phosphorylation (reviewed in Jope, 2003; Rowe *et al*. 2007). Therefore, investigating treatments that target GSK-3-linked pathways is a rational strategy for developing novel therapeutics to treat this disorder.

GSK-3 abnormalities have been implicated in the pathophysiology of various mood disorders. For example, decreased Akt activity and increased GSK-3 activity were noted in the post-mortem prefrontal cortex of depressed individuals who committed suicide (Karege *et al*. 2007). Using the forced swim test, chronic treatment with lithium produces antidepressant-like effects in mice, reminiscent of its clinical efficacy in patients with bipolar disorder (O'Brien *et al*. 2004). Other studies have noted that GSK-3 peptide inhibitors

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(Kaidanovich-Beilin *et al*. 2004) and novel GSK-3 inhibitors (Gould *et al*. 2004) also induce rapid antidepressant-like and/or antimanic-like effects in mice, suggesting that GSK-3 is a potential mood-stabilizing target of lithium. In addition, target deletion of *GSK-3β* gene to produce heterozygous GSK-3$\beta^{+/-}$ mice produces behavioural effects similar to the antidepressant-like effects of lithium (O'Brien *et al*. 2004). However, to date the brain region(s) responsible for these drug-induced antidepressant-like effects remain elusive. In this context, chronic lithium treatment has been shown to have brain region-selective neuroprotective effects in rodents (Omata *et al*. 2008), and these neuroprotective effects may contribute to its clinical efficacy (van der Schot *et al*. 2009). Thus, it is unlikely that the antidepressant-like effects of GSK-3 inhibitors are mediated by actions occurring throughout the whole brain.

RNA interference (RNAi) has become an effective tool for selectively silencing the expression of GSK-3 isoforms and for elucidating GSK-3 isoform-associated neurobiological functions (Liang & Chuang, 2006; Liang & Chuang, 2007). We elucidated differences in GSK-3 isoforms where GSK-3β depletion is more effective than GSK-3α depletion in suppressing spontaneous cell death in extended culture (Liang & Chuang, 2007). While depletion of both isoforms was able to block glutamate-induced-excitotoxity and warrant further investigation, we focused on GSK-3β in the present study. Lentiviral vectors have been developed to effectively and safely transfer genes into dividing and non-dividing cells such as post-mitotic neurons. For example, the lentiviral vector that expresses a short-hairpin RNA (shRNA) inhibits the expression of target proteins specifically and persistently *in vitro* and in the rat brain (Sapru *et al*. 2006). Furthermore, the hippocampal dentate gyrus has been implicated as one of the brain regions involved in modulating the efficacy of antidepressants via mechanisms involving brain-derived neurotrophic factor (BDNF) (Adachi *et al*. 2008; Shirayama *et al*. 2002). In the present study, we used chronic restraint stress as a mouse model of depression. We examined the antidepressant-like effects of persistent GSK-3β inhibition induced by injection of lentiviral vectors expressing GSK-3β shRNA into the hippocampal dentate gyrus of these mice.

Materials and methods

shRNA design

GSK-3β Mission® shRNA plasmid (PLK 0.1 puro-GSK-3β-shRNA) and non-targeting shRNA control vector (PLK 0.1 puro-control shRNA) were purchased from Sigma (St Louis, USA). shRNA was designed against GSK-3β mRNA, and the sequence was 5′-CCGGCCACTCAAGAACTGTCAAGTACTCGAGTACTTGACAGTTCTTGAGTGGTTTTT-3′. The control vector produced a corresponding scrambled shRNA, with a sequence of 5′-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3′.

Lentiviral vector production and concentration

HEK-293 T/17 cells were grown in DMEM/10% FBS and plated in 10-cm dishes at a density of 1×10^6 cells/dish. The day after plating, GSK-3β Mission® shRNA plasmids were transfected as follows: 2.6 μg GSK-3β plasmid DNA, 26 μl lentiviral packaging mix (Sigma), and 16 μl FuGENE transfection reagent (Roche, USA) per dish in 10 ml DMEM. The day after transfection, culture medium was collected and immediately frozen for preparation of GSK-3β-containing particles. For the second harvest of viral particles, DMEM was replaced with fresh medium for another collection on the next day. Collected medium was filtered (0.45 μm pore) and ultracentrifuged at 4 °C for 90 min at 25 000 rpm. The pellet was resuspended with 200 μl PBS and left standing for 30 min at 4 °C. Viral particles from three tubes were collected, and concentrated by ultracentrifugation. Viral titre was detected using an HIV-1 p24 Antigen ELISA kit (ZeptoMetrix Corporation, USA), and the final injection titre was 2.5×10^8 TU/ml.

Animals, stereotaxic surgery, and antidepressant treatment

Animal procedures were approved by the Animal Care and Use Committee of the National Institute of Mental Health, National Institutes of Health (Bethesda, USA). Male CD-1 mice aged 8 wk were bred and housed at 24 ± 1 °C under a 12-h light/dark cycle (lights on 07:00 hours), with food and water available *ad libitum*. Animals were anaesthetized intraperitoneally with ketamine (80 mg/kg) and xylazine (8 mg/kg), and then mounted onto a stereotaxic apparatus (Stoelting Instruments, USA). One microlitre of lentivirus was injected targeting both sides of the hippocampal dentate gyrus at a rate of 0.25 μl/min using a 25-gauge Hamilton syringe. Coordinates of injection site were: AP −2.0, ML ±1.6 relative to bregma, and DV −2.0 relative to the skull surface (Paxinos & Franklin, 2001). The needle was left in place for an additional 4 min and then withdrawn. For the measurement of the behavioural effects of a reference antidepressant, desipramine hydrochloride

(Sigma) was freshly dissolved in deionized water before use and injected intraperitoneally 30 min prior to behavioural testing in a volume of 10 ml/kg of body weight. Previous studies have shown that CD-1 mice are sensitive to the behavioural effects of desipramine, and 20 mg/kg of this drug markedly reduces the immobility time in the forced swim test (Lucki *et al*. 2001). Control animals received injection of deionized water as the vehicle.

Chronic restraint stress

Animals recovered for 7 d after surgery before initiation of chronic restraint stress, which was performed as previously described (Pawlak *et al*. 2003) with minor modifications. Briefly, each mouse was placed into a Plexiglas tube (diameter 2.5 cm) without access to either food or water for 2 h a day for 14 consecutive days. Open-field testing (see below) was conducted 1 d after chronic restraint stress ended. Two days after the last chronic restraint stress, mice were given either the forced swim test or the tail suspension test. Following evaluation of behavioural performance, animals were sacrificed for GSK-3β analysis.

Open-field test

Briefly, as previously described (Fukui *et al*. 2007), each mouse was placed in the centre of an open field ($27 \times 27 \times 20$ cm) with a white floor and clear plastic walls, equipped with infrared sensors. Testing lasted for 30 min. Distance travelled and average velocity were measured using Activity Monitor software version 5 (Med Associates Inc., USA).

Forced swim test

Briefly, as previously described (Porsolt *et al*. 1977), each mouse was placed for 6 min in a Plexiglas cylinder (height 25 cm, diameter 15 cm) containing 20 cm water maintained at 25 ± 1 °C. Sessions were digitally recorded for later analysis. Duration of immobility, defined as lack of activity, except movements made by mice to keep their heads above water, was scored during the last 4 min.

Tail suspension test

Briefly, as previously described (Steru *et al*. 1985), each mouse was suspended 30 cm from the floor by the distal portion of its tail with adhesive tape for 6 min. Sessions were digitally recorded and later scored for duration of immobility – defined as absence of active attempts to escape – from the beginning to the end of the test.

Western blotting

Both hippocampi were isolated and homogenized in lysis buffer. Protein concentrations were determined and 10-μg aliquots were separated by electrophoresis on 4–12% Nupage Bis-Tris gel. Proteins were subsequently transferred to a polyvinylidene difluoride membrane, incubated with primary antibody against GSK-3β (1:5000; BD Biosciences, USA), phospho-Ser9-GSK-3β (1:1000; Cell Signaling, USA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Advanced Immunochemical, USA). The membrane was washed with 0.1% Tween PBS, and then incubated with horseradish peroxidase-labelled secondary antibody (1:2000; GE Healthcare Bioscience Corp., USA). Reactive bands were visualized by detecting chemiluminescence and quantified by using an FLA-7000 imaging system and Multi Gauge software version 3.0 (Fuji Photo Film Co. Ltd, Japan).

Immunohistochemistry

Immunohistochemical analysis was performed using a R.T.U. Vectastain kit (Vector Laboratories, USA). Mice were anaesthetized and perfused through the left cardiac ventricle with normal saline followed by 4% paraformaldehyde. Brains were removed and cut using a cryostat (Leica Microsystems Inc., USA) at a thickness of 30 μm, and incubated with primary antibody against GSK-3β (1:1000; BD Biosciences). Brain slices were washed with TBS, and incubated with horseradish peroxidase-combined secondary antibody. Immunostained brain slices were further washed with TBS, and then developed with ImmPACT DAB substrate (Vector Laboratories).

Statistical analysis

All data are presented as mean ± S.E.M. For comparison between two groups, Student's *t* test was used. For comparison of more than three groups, statistical analysis was done by one-way analysis of variance (ANOVA) followed by *post-hoc* Student–Newman–Keuls multiple comparison tests. A p value < 0.05 was considered statistically significant.

Results

Effects of intra-hippocampal injection of lentiviral-based GSK-3β shRNA on behavioural performance in chronically stressed mice

In mice undergoing a model of stress-induced depression, lentiviral vector-expressing shRNA of GSK-3β was injected bilaterally into the hippocampal

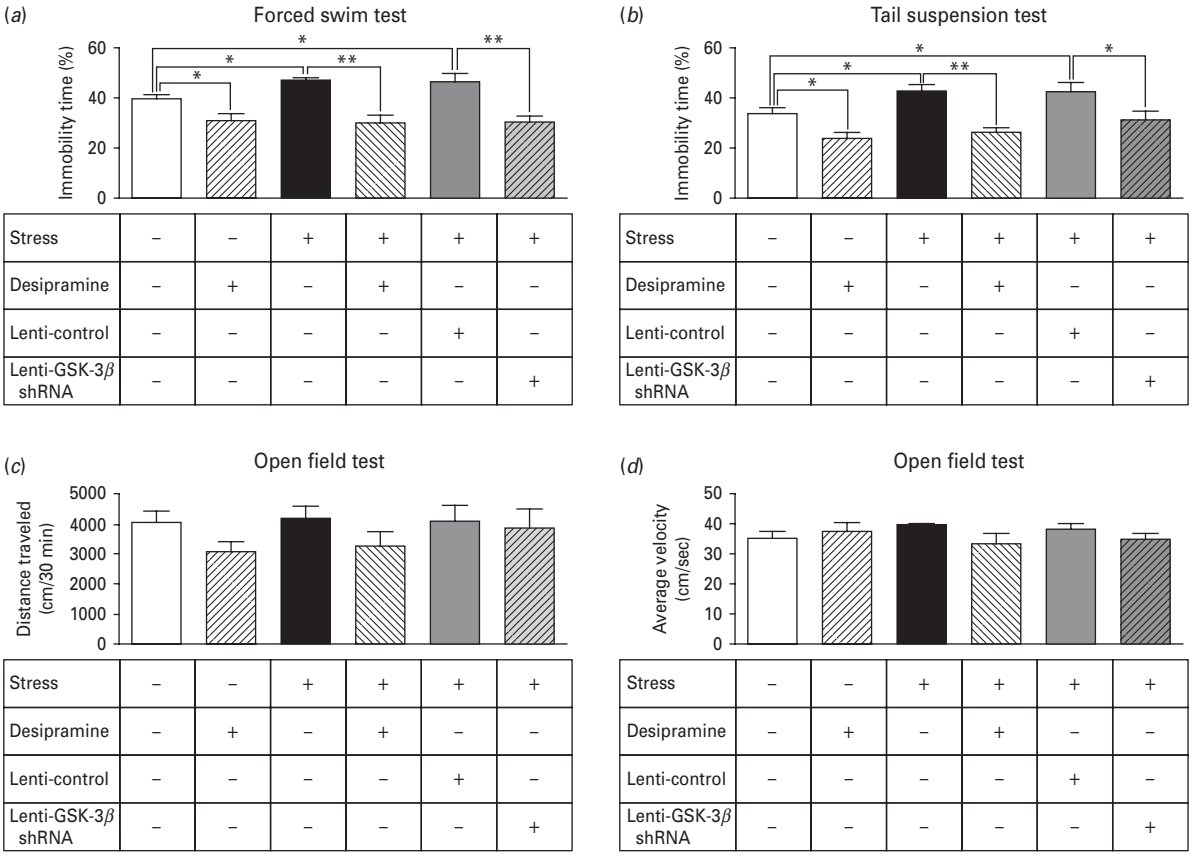

Fig. 1. Intra-hippocampal injection of lentivirus-expressing GSK-3β shRNA produced an antidepressant-like effect, but did not affect locomotor activity, in chronically stressed mice. Intra-hippocampal injection of lentivirus-expressing GSK-3β shRNA or intraperitoneal pretreatment with desipramine decreased immobility time in (*a*) the forced swim test and (*b*) the tail suspension test. Data are mean $\pm$ S.E.M. of immobility time expressed as percentage of the test session (%, $n = 6$–10). * $p < 0.05$, ** $p < 0.01$, by Student–Newman–Keuls multiple comparison test after one-way ANOVA. Locomotor activity was measured by the open-field test and expressed as (*c*) the distance travelled and (*d*) average velocity.

dentate gyrus 7 d prior to beginning the stress procedure in order to induce a persistent reduction of GSK-3β expression levels. Mice subsequently underwent the forced swim and tail suspension tests to assess any associated antidepressant-like effects. Compared to drug-naive, non-stressed mice, chronic restraint stress markedly increased the immobility time in the forced swim test (from 39.81 ± 1.54% to 47.13 ± 1.03%, $n = 8$). This depressive-like behaviour was suppressed by 30-min pretreatment with 20 mg/kg of an antidepressant, desipramine. Similar to the effect produced by this antidepressant, injection of the lentivirus-expressing GSK-3β shRNA significantly reduced immobility time by approximately 35% compared to the group injected with the control lentiviral vector after chronic restraint stress (from 46.61 ± 3.39% to 30.47 ± 2.39%, $n = 8$) (Fig. 1*a*). Similarly, pretreatment of stressed mice with desipramine decreased the immobility time in the tail suspension test. A significant decrease in immobility time was also detected in mice injected with the lentivirus-expressing GSK-3β shRNA compared to those injected with the control lentiviral vector (from 42.83 ± 3.68% to 31.48 ± 3.34%, $n = 6$) (Fig. 1*b*). In contrast, locomotor activity (expressed as distance travelled and average velocity) assessed by the open-field test after chronic stress was unchanged by either desipramine or lentiviral GSK-3β shRNA injection (Fig. 1*c*,*d*). No difference in the temporal profiles of locomotor activity throughout the 30-min open-field test was observed between different experimental groups (Supplementary Fig. S1, available online). These results suggest that intra-hippocampal injection of lentivirus-expressing GSK-3β shRNA affected neither basal locomotion nor the habituation of mice to a novel environment.

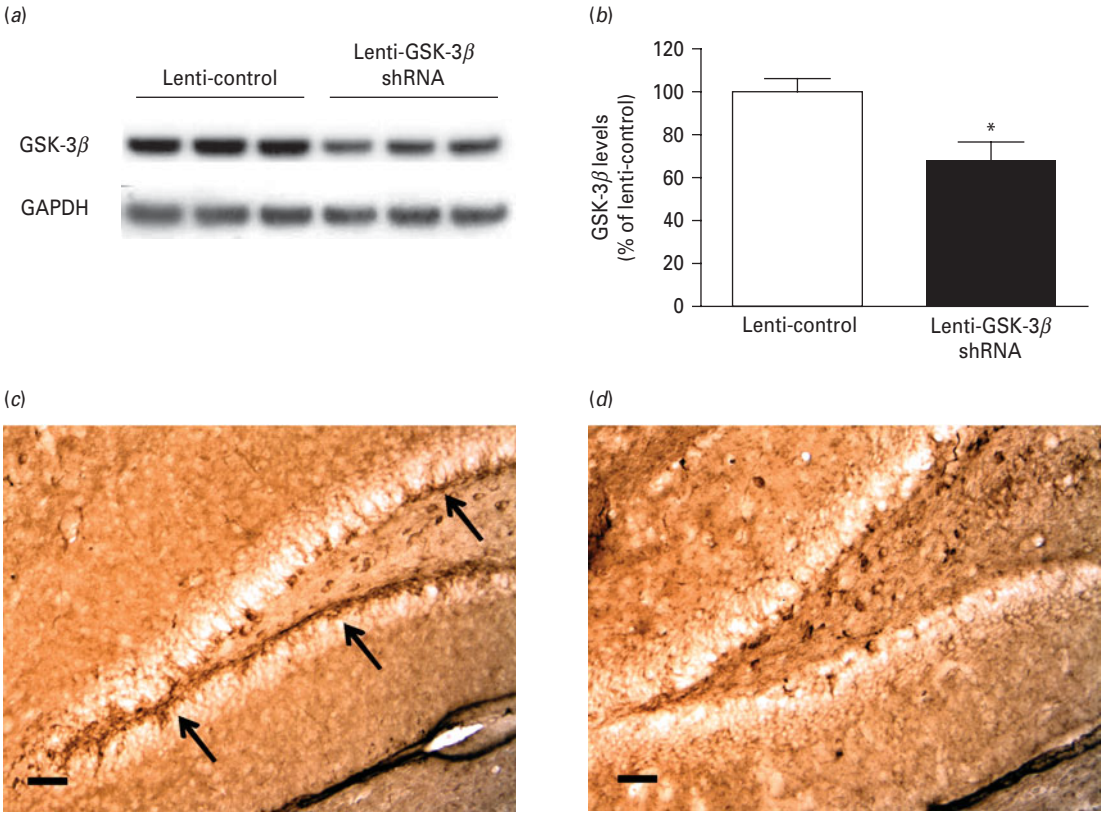

Fig. 2. Protein levels and immunohistochemical staining of GSK-3β in the hippocampus were decreased by injection of lentiviral GSK-3β shRNA into the dentate gyrus. (*a*) Western blots of GSK-3β and GAPDH (used as a loading control) in the entire hippocampus from three mice randomly selected from each group. (*b*) Quantified results of the blots of GSK-3β shown in panel (*a*) ($n=6$). * $p<0.05$ compared to control. Typical results of GSK-3β immunostaining in the hippocampal dentate gyrus of a mouse injected with (*c*) a control vector or (*d*) lentiviral GSK-3β shRNA. Arrows indicate positive staining of GSK-3β-expressing cells. Scale bar, 50 μm.

Effects of intra-hippocampal injection of lentiviral-based GSK-3β shRNA on GSK-3β expression levels in the hippocampus

Animals were sacrificed after assessment of behavioural performance, in order to perform Western blotting for GSK-3β protein in the hippocampus. We found that total hippocampal GSK-3β protein levels were decreased by about 30% following injection of the lentiviral vector expressing GSK-3β shRNA, compared to the group injected with the control vector (Fig. 2*a*, *b*). Furthermore, immunostaining results showed that GSK-3β was clearly detected in the hippocampal dentate gyrus, particularly in the subgranular zone in the control group (Fig. 2*c*). However, GSK-3β immunostaining in the dentate gyrus was robustly reduced by injection with the lentivirus-expressing GSK-3β shRNA (Fig. 2*d*).

Discussion

To the best of our knowledge, this study is the first to use local injections of lentivirus-expressing shRNA to specifically knockdown GSK-3β in the hippocampal dentate gyrus and to assess any associated behavioural changes. Notably, we found that a single injection of lentivirus-expressing GSK-3β shRNA in the hippocampal dentate gyrus elicited significant antidepressant-like effects in mice undergoing a chronic stress-induced animal model of depression.

In the present study, total hippocampal GSK-3β protein levels were reduced to about 70% compared to control animals. This modest but significant knockdown of hippocampal GSK-3β may be explained by the use of the whole hippocampus for Western blotting, where the inclusion of non-transduced tissue dilutes the knockdown effect of the procedure.

Nevertheless, the GSK-3β knockdown was sufficient to elicit changes in behaviours revealed in our tests. A previous report indicates that chronic stress elevates GSK-3β mRNA in the hippocampus (Silva *et al*. 2008). We found that hippocampal GSK-3β protein levels were not significantly increased by stress; however, its GSK-3β Ser9 phosphorylation was significantly reduced, thus indicating enhanced GSK-3β activity (Supplementary Fig. S2, online). These data are reminiscent of the recent report that brain GSK-3β Ser9 phosphorylation is decreased in the learned helpless state after inescapable foot shock (Polter *et al*. 2010). We also showed that GSK-3β immunostaining was robustly reduced in the dentate gyrus of stressed mice injected with lentivirus-expressing GSK-3β shRNA. Our results support the notion that lentiviral-mediated RNAi effectively disrupts region-selective gene expression for rapid functional analysis, thus circumventing the need to generate gene knockout animals.

Notably, treatment with lentivirus-expressing GSK-3β shRNA significantly reduced the immobility time of the mice in the forced swim and tail suspension tests. These two tests are widely used for screening antidepressant activity, and reduced immobility time is considered a well-validated antidepressant-like effect (Porsolt *et al*. 1977; Steru *et al*. 1985). Because false-positive results can be obtained in these tests due to stimulation of locomotor activity (Bourin *et al*. 2001), we also assessed this measure in our study and found that lentiviral GSK-3β shRNA had no effect on locomotor activity. Thus, our results suggest that knockdown of GSK-3β produces antidepressant-like effects.

Previous studies have noted that GSK-3 inhibitors have acute antidepressant-like and/or antimanic-like effects in naive rodents (Gould *et al*. 2004; Kaidanovich-Beilin *et al*. 2004). However, the lack of clear selectivity of these inhibitors for GSK-3 among various kinases has made it difficult to reliably assess the link between GSK-3 inhibition and mood-stabilizing effects (Hongisto *et al*. 2008). In addition, these inhibitors probably block the activity of both GSK-3α and GSK-3β; thus, the role that each individual GSK-3 isoform plays in mediating behavioural effects has to date remained unclear. Furthermore, neither pharmacological studies nor experiments using GSK-3$\beta^{+/-}$ knockout mice (O'Brien *et al*. 2004) provide information regarding which brain region is critically involved in GSK-3's antidepressant-like effects. In contrast, lentiviral-mediated RNAi provides an alternative approach where expression of a particular GSK-3 isoform can be silenced selectively and persistently in a specific brain region for behavioural evaluations. We believe our results are the first to demonstrate that administration of lentiviral-mediated GSK-3β shRNA into the dentate gyrus causes antidepressant-like effects in mice subjected to chronic stress.

While numerous brain regions have been suggested to contribute to the pathophysiology of mood disorders, previous studies have strongly implicated the hippocampal dentate gyrus and this brain region has been the focus of our investigation. Patients with major depressive disorder have reduced hippocampal volume (McKinnon *et al*. 2009). Infusion of BDNF into the dentate gyrus has been found to induce antidepressant-like effects in rodents (Shirayama *et al*. 2002), and selective loss of BDNF in the dentate gyrus, but not the CA1 region, was shown to attenuate the actions of antidepressant drugs in the forced swim test (Adachi *et al*. 2008). In addition, adult neurogenesis occurs in the hippocampal dentate gyrus; this event is facilitated by hippocampal BDNF infusion (Scharfman *et al*. 2005) which is also crucial for the sensitivity to antidepressant treatment (Li *et al*. 2008). Interestingly, recent work from our laboratory showed that selective knockdown of GSK-3β using specific siRNA induced BDNF expression via transcriptional activation of promoter IV in rat cortical neurons (Yasuda *et al*. 2009). Therefore, it is conceivable that the antidepressant-like effects elicited in this study by intra-hippocampal injection of lentiviral GSK-3β shRNA involve BDNF induction and subsequent enhanced neurogenesis in this brain region. Further studies are needed to address whether injection of lentiviral GSK-3β shRNA into the dentate gyrus or other brain region(s) has antimanic effects similar to those seen in individuals with bipolar disorder who receive chronic lithium therapy. The role of the GSK-3α isoform in mediating mood states also remains to be investigated. Nevertheless, our findings provide evidence that persistent silencing of GSK-3β expression in the hippocampal dentate gyrus is sufficient to induce antidepressant-like behaviours in stressed mice.

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Statement of Interest

None.

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