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GSK3ß, a centre-staged kinase in neuropsychiatric disorders, modulates long term memory by inhibitory phosphorylation at Serine-9

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ABSTRACT

Accumulating evidence implicates deregulation of GSK3ß as a converging pathological event in Alzheimer's disease and in neuropsychiatric disorders, including bipolar disorder and schizophrenia. Although these neurological disorders share cognitive dysfunction as a hallmark, the role of GSK3ß in learning and memory remains to be explored in depth. We here report increased phosphorylation of GSK3ß at Serine-9 following cognitive training in two different hippocampus dependent cognitive tasks, i.e. inhibitory avoidance and novel object recognition task. Conversely, transgenic mice expressing the phosphorylation defective mutant GSK3ß[S9A] show impaired memory in these tasks. Furthermore, GSK3ß[S9A] mice displayed impaired hippocampal L-LTP and facilitated LTD. Application of actinomycin, but not anisomycin, mimicked GSK3ß[S9A] induced defects in L-LTP, suggesting that transcriptional activation is affected. This was further supported by decreased expression of the immediate early gene *c-Fos*, a target gene of CREB. The combined data demonstrate a role for GSK3ß in long term memory formation, by inhibitory phosphorylation at Serine-9. The findings are fundamentally important and relevant in the search for therapeutic strategies in neurological disorders associated with cognitive impairment and deregulated GSK3ß signaling, including AD, bipolar disorder and schizophrenia.

Introduction

A potential pathological role is emerging for glycogen synthase kinase-3 (GSK3) in the most prevalent neurodegenerative disorder, i.e. Alzheimer's disease (AD) (Muyllaert et al., 2008; Terwel et al., 2008; Hooper et al., 2008; Small and Duff, 2008). A growing body of evidence implicates GSK3 in other neuropsychiatric disorders, i.e. bipolar disorder, depression, schizophrenia, anxiety disorder (Gould et al., 2004, Guzzetta et al., 2007, Lovestone et al., 2007). While these neurological diseases vary in symptoms, age of onset and systems affected, they share cognitive dysfunction and derangement of thoughts as common feature.

GSK3 is a proline-directed serine/threonine kinase, originally identified as a regulator of glycogen metabolism (Embi et al., 1980). Mammals express two isozymes encoded by different genes (GSK3α and GSK3ß) (Woodgett, 1990) that are inactivated by phosphorylation at S21/S9 respectively, by various protein kinases (Frame and Cohen, 2001). Numerous substrates of GSK3 include transcription factors (e.g. CREB), factors involved in translational control (e.g. eIF2 family), metabolic enzymes, cytoskeletal and motor proteins, components involved in cell division and cell adhesion (Frame and Cohen, 2001; Doble and Woodgett 2003; Jope and Johnson, 2004).

Deregulation of GSK3 is considered important in schizophrenia and bipolar disorder (Lovestone et al., 2007; Gould et al., 2004). Atypical and typical antipsychotic drugs alter GSK-3 activity, as do drugs that induce psychosis. GSK-3 regulatory pathways are altered in these disorders, and associated genes directly or indirectly regulate GSK-3 activity (Lovestone et al., 2007, Gould et al., 2004; Einat and Manji, 2006). Cognitive impairment is considered as a core feature to bipolar disorder and schizophrenia and considered "endophenotypic" in genetic studies (Einat and Manji, 2006; Powell and Miyakawa, 2006). Importantly, functional outcome in schizophrenia and bipolar disorder are correlated to and even predicted by cognitive dysfunction (Green et al., 2006; Burdick et al., 2007; Martinez-Aran et al., 2007). Therapeutic strategies targeting cognitive impairment in schizophrenia and bipolar disorders are gaining ground (Burdick et al., 2007; Martinez-Aran et al., 2007).

GSK3ß is centre-stage in the pathogenesis of Alzheimer's disease as Tau kinase I phosphorylating Tau at epitopes that mark neurofibrillary tangles (review Muyllaert et al., 2006; Hooper et al., 2008). GSK3ß is associated with neurofibrillary tangles and its activity is increased in brain of AD patients (Leroy et al., 2007). GSK3ß

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phosphorylates neuronal Tau in mouse brain in vivo (Spittaels et al., 2000, Lucas et al., 2001, Engel et al., 2006), while its co-expression with TauP301L dramatically aggravates neurofibrillary pathology (Terwel et al., 2008). Finally, in brain of APP transgenic mice GSK3 is activated (Muyllaert et al., 2006; Terwel et al., 2008).

Here we demonstrate a central function for GSK3ß in long term memory by two complementary data-sets: (i) increased phosphorylation at GSK3ß-S9 following cognitive training in hippocampus dependent tasks and (ii) constitutively active GSK3ß[S9A] impaired long term memory in vivo and ex vivo (i.e. late long term potentiation (L-LTP) while facilitating long term depression (LTD)). Additional pharmacological and biochemical evidence suggest an important role for transcriptional regulation by GSK3ß. The combined data imply a crucial function for GSK3ß in long term memory and a role in cognitive dysfunction in GSK3 related neurological disorders.

Materials and methods

Animal housing and behavioral procedures

Adult GSK3ß[S9A] transgenic mice and non-transgenic littermates, age and sex-matched (4–6 months) were used in all experiments. Animals were housed on a 12-hour light/dark cycle in standard animal care facilities and acclimatized in a vivarium at least 6 days prior to behavioral assessment, with access to food and water *ad libitum* (except for conditioned taste aversion task). All experiments are approved and overseen by the KULeuven Ethical Committee for Animal Welfare (KUL-ECD).

Mice

Transgenic mice expressing the phosphorylation defective, constitutively active GSK3ß[S9A] were generated by classical microinjection of its cDNA ligated in the adapted mouse thy1 gene promoter as described (Spittaels et al., 2000). The mice display increased GSK3ß activity and increased protein Tau phosphorylation (Spittaels et al., 2000, Terwel et al., 2008, Muyllaert et al., 2008). Genotyping was performed on tail biopsy DNA by polymerase chain reaction.

Behavioral testing

Open field test

Mice were placed in a corner of the open field perspex box ($52\times52\times40$ cm) with black vertical walls and translucent floor, dimly illuminated from below. Activity of individual mice over 10 min observation periods was recorded and analyzed by a dedicated computerized system (EthoVision, Noldus, Wageningen, The Netherlands). The open field was divided in virtual sections using dedicated software, i.e. center, periphery, and corner zones, defined by parallel lines 10 cm from the walls. Behavioral parameters that were calculated include total distance traveled, speed, number of entries and residence time in each section.

Light/dark exploration test

The light/dark exploration test was performed by placing the mouse in a cage ($52\times52\times40$ cm) divided into 2 compartments, 1 brightly lit from above and 1 dark. Mice were initially placed in the dark compartment, and observed for 5 min to record the time spent in each section, as well as the number of transitions between dark/light compartments.

Inhibitory avoidance task

On the day of training, mice were transferred to the sound-attenuated room with the IA training apparatus, i.e. a two-chambered box with lit and dark sections, separated by a trap-door. For training, mice were initially placed in the lit side of the box facing a corner opposite the door. After 10 s the trap-door was opened to allow the mouse to enter the dark section, where they received an electric footshock after 2 s (mild stimulation: 0.5 mA; 1 s; strong stimulation 1 mA; 2 s). The mice were allowed 15 s to recover in the dark compartment before being returned to the home cage. At defined intervals after training, the mice were euthanized for analysis, as specified with the results. Retention was assessed by placing mice in the lit section and measuring the latency of re-entry into the dark compartment 24 h after training, with the criterion of all 4 paws present in the dark section of the IA apparatus. The retention index was calculated as the increase in latency after training ($100\times$(latency in test trial/latency in training trial)). Statistical significance for avoidance retention was determined using a single factor ANOVA, with the criterion for significance at $p<0.05$.

Novel object recognition task

The novel object recognition task was essentially performed as described (Dewachter et al., 2002, Rampon et al., 2000, Tang et al., 1999). Briefly, mice were habituated for 10 min in the perspex open field box dimly illuminated from below the box (see above). The following day, mice were observed in the same box for the 8 min acquisition trial in the presence of object A. The time was recorded that the mice explored object A, with as criterion that the snout was directed towards object A at less than 1 cm. The exploration time was used as a measure for explorative behavior of the mouse. The 8 min retention trial was performed 4 h later, by placing the mouse in the box with an additional, novel object B besides the familiar object A. The length of time (tA and tB) that the animal spent exploring both objects was recorded. The retention index (RI) was defined as the ratio of the time spent exploring the novel object over the time spent exploring both objects $[(tB/(tA+tB))\times100]$. Statistical analysis was performed by ANOVA single factor as described previously.

Contextual and cued fear conditioning

Contextual and cued fear conditioning was performed in a standard chamber with grid floor connected to electric output device (Med Associates, St. Albans, VT). The training consisted of a single trial, whereby after a 60 s baseline period mice were exposed to 3 tone (CS)-footshock (US) pairings (tone (CS), 30 s, 3000 Hz, 90 dB; footshock (US), 2 s, 0.5 mA; intertrial interval, 2 min; CS and US co-terminated at the end of the training block). Ninety seconds after the last shock, mice were returned to their home-cages. After 24 h, contextual fear conditioning was assessed by returning the mice to the conditioning chamber during a 5 min period. In cued fear conditioning, the conditioning stimulus (CS) (90 dB; 3000 Hz) was presented for 2 min, starting 3 min after the mouse was placed in the novel environment created by covering floor and side-walls with white perspex tiles. Freezing was quantified automatically using video-based conditioned fear testing system (FreezeFrame; Actimetrics, Evanston, IL). Freezing data are presented as percent time spent freezing, i.e. time spent freezing/total time$\times100$). All data are expressed as mean $\pm$ S.E.M.

Conditioned taste aversion

Conditioned taste aversion was performed with mice that were deprived from drinking water *ad libitum*, but were trained to drink during two daily sessions of 30 min each with a 4-hour interval for 4 days. Water was presented in two identical 15-ml vessels, that were weighed before and after the test as measure of water intake. During the morning session on the conditioning day (day 5) mice were allowed to drink a 0.5% saccharin solution as conditioning stimulus, and 30 min later mice were injected intraperitoneally with LiCl (4.5 mmol/kg) in saline as the nausea-inducing agent as unconditioned stimulus. In the next session after a 2-hour interval, mice were allowed to drink water before being returned to their home cage. During the 30-minute test session, 24 h later, the mice were presented

with two identical vessels with either water or 0.5% saccharin-water solution and the volume of consumed fluid was measured for each, to calculate the aversion index as $V_{water} / V_{water} + V_{saccharin}$.

Biochemical analysis

At indicated time-points following cognitive training, mice were euthanized rapidly by cervical dislocation, and hippocampi were dissected rapidly in the cold. Hippocampi were homogenized in 6 volumes of ice-cold homogenization buffer (25 mM Tris.HCL, 150 mM NaCl, pH 7.6) containing complete protease and phosphatase inhibitor cocktails (1 mM EGTA, 30 mM NaF, 2 mM Na_3VO_4, 1 μM okadaic acid, 1 mM PMSF) (Terwel et al., 2008) in a Potter–Elvejhem homogenizer with 10 up-and-down strokes of a Teflon pestle rotating at 700 rpm. Protein concentrations were measured using the BCA protein assay. Homogenates were diluted appropriately in sample buffer for SDS-PAGE on 8% polyacrylamide gels (Novex, San Diego, CA). Equal loading was demonstrated by western blotting with anti-tubulin antibody revealing no significant differences between experimental groups (cfr representative blots Fig. 1S M&M). Homogenates of brains of GSK3ß[S9A] mice and non-transgenic littermates were prepared with the same procedure. Western blotting was performed as described (Dewachter et al., 2000) using primary antibodies detecting GSK3ß (Transduction Laboratories, BD Biosciences, CA), GSK3ß phosphorylated at Serine-9 (Cell Signalling, Boston, MA) and c-Fos (Santa-Cruz, CA). Quantitation by densitometric scanning of exposed films was performed on a flatbed optical density scanner and dedicated software (Image Master, GE-Health, Uppsala, Sweden).

Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously (Dewachter et al., 2002). Brains were dissected and the right cerebral hemisphere was immersion fixed in 4% paraformaldehyde in PBS overnight for histological analyses. Immunohistochemistry with anti-NeuN mouse monoclonal antibody (Chemicon/Millipore, MA), anti-CD45 rat antibody (BD Biosciences, CA) and anti-GFAP rabbit polyclonal antibody (DAKO, Haasrode, Belgium) was done on free-floating saggital vibratome sections using diaminobenzidine as chromogen. Staining with FluoroJadeB was performed to monitor neurodegeneration on free-floating saggital vibratome sections (40 μm) mounted on gelatin-coated glass-slides and air-dried. Dehydration was by passage through a graded series of ethanol (100, 75, 50, and 25%; 5 min each).Sections were washed 2 min in 70% ethanol, followed by 2 min in water, and 2 min incubation in 0.06% potassium permanganate solution. After incubation with Fluorojade B solution (0.0004%) for 10 min), the slides were washed 3 times in water (2 min), dried for 10 min at 50 °C before mounting with Depex.

Electrophysiological measurements

Hippocampal sections (400 μm) were kept and perfused with artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 5 mM KCl, 26 mM NaHCO3, 1.24 mM NaH2PO4, 2.5 mM CaCl2, 1.3 mM MgSO4, 10 mM glucose) aerated with 95% O2/5% CO2 gas mixture. Sections were placed in the interface recording chamber and kept at 28 °C for 90 min with perfusion with ACSF (1 ml/min). Bipolar twisted nickel-chrome electrodes (50 μm) were used to stimulate Schaffer's collaterals, while extracellular field excitatory postsynaptic potentials (fEPSP) were recorded in the CA1 stratum radiatum, using low resistance (2–5 mΩ) glass electrodes filled with ACSF. Test stimuli were biphasic (0.08 ms) constant-voltage pulses delivered every minute with intensity adjusted to evoke approximately 40% maximal responses. The slope of the fEPSP was averaged over 4 consecutive responses. LTP was induced either by weak stimulation, i.e. single train of pulses at 100 Hz for 1 s, or by strong stimulation, i.e. 4 trains at 100 Hz of 1 s with 5 min

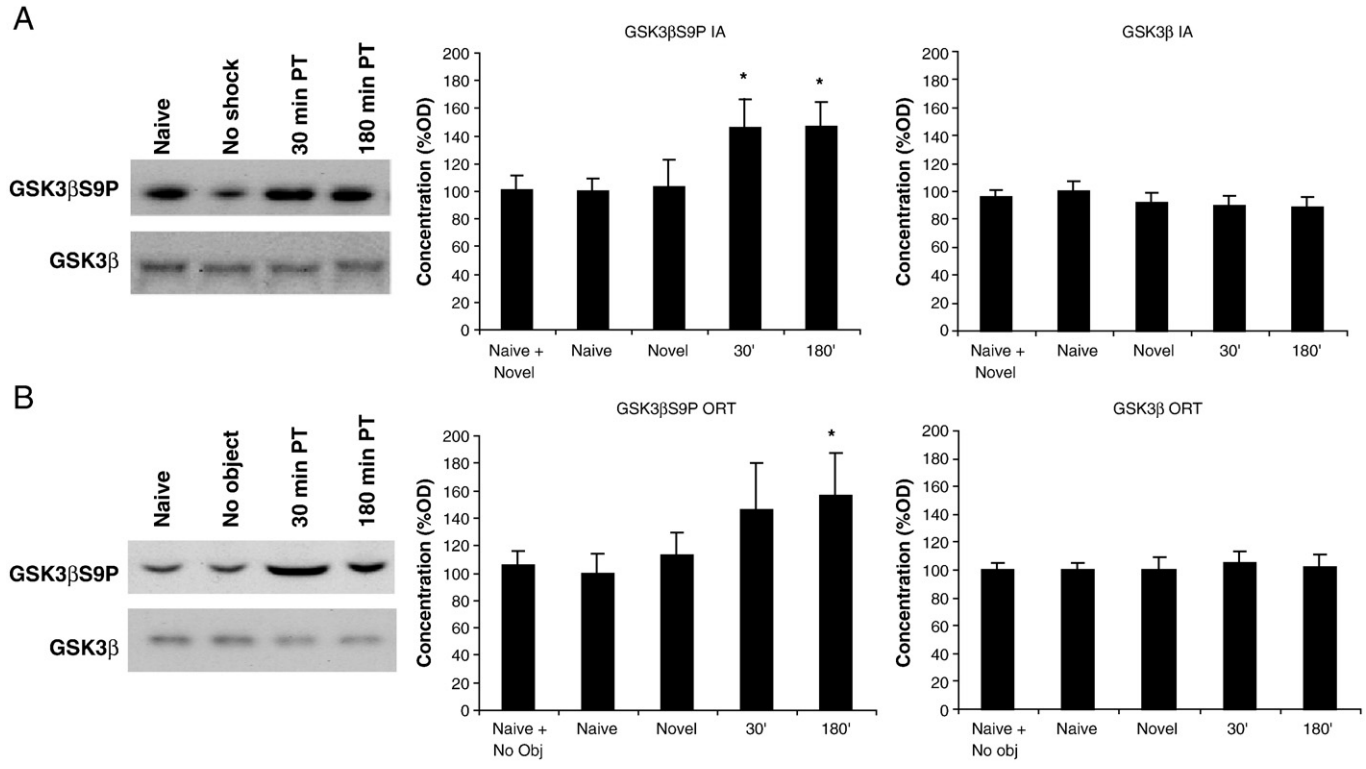

Fig. 1. Cognitive training increases GSK3ß phosphorylation of S9 in wild-type mice. (A) Increased phosphorylation of GSK3ß at S9 in the hippocampus of wild-type mice 30 and 180 min post-training (PT) in the inhibitory avoidance task, relative to non-trained wild-type mice ($n = 5$ for each group; *$p < 0.05$). Protein levels of total GSK3ß were not significantly affected by training. Naive mice and mice handled without shock were not significantly different. (B) Increased GSK3ß-S9 phosphorylation in the hippocampus of wild-type mice 180 min post-training (PT) in the novel object recognition task, in comparison to naive mice ($n = 5$–7; *$p < 0.05$). Naive mice and mice handled without object presentation were not significantly different.

intervals, with subsequent measurement of the potentiated response for respectively 2 and 4 h. The fEPSP during stimulation was recorded and for each section, the fEPSP slopes were normalized with respect to the mean recorded for 30 min prior to LTP-induction. To determine whether or not normalized fEPSP of a group of sections assessed in identical experimental conditions was significantly potentiated ($p<0.05$), the relative baselines measured immediately before, and at 3 and 4 h after LTP-induction were compared by paired Student's *t*-test. Statistical significance ($p<0.05$) of the differences in fEPSP after LTP-induction in two groups of slices was assessed by a Student's *t*-test. For each slice, an input–output curve was established by increasing stimulus intensity. Synaptic fatigue was analyzed by measuring output during high frequency stimulation. Paired-pulse facilitation was tested at four different time intervals: 50 ms, 100 ms, 150 ms and 200 ms, and measured by the relative ratio of the slope of the second to the first fEPSP. LTD was induced by delivering 900 pulses of 1 Hz. Following collection of a stable baseline recording, stimulation was delivered to induce LTD and the responses were followed for 2 h. Changes in synaptic strength were quantified by normalizing the slope of the population excitatory postsynaptic potential (fEPSP) for each experiment by the mean EPSP slope at baseline set at 100%, and changes are expressed relative to this baseline. Statistical significance ($p<0.05$) of the differences in fEPSP after LTD-induction was assessed by a Student's *t*-test.

Statistical analysis

Data were statistically analyzed using methods of distribution statistics (standard descriptive analysis), analysis of the means (Student *t*-test) and analysis of variance (ANOVA). A probability of $p<0.05$ was considered significant. All data are expressed as mean ± S.E.M.

Results

Cognitive training induces inhibitory phosphorylation of GSK3ß at Serine-9

To analyze modulation of GSK3ß by cognitive training, phosphorylation of GSK3ß-S9 was measured following learning in the inhibitory avoidance (IA) paradigm, a rapidly acquired, stable, hippocampus dependent type of memory (Whitlock et al., 2006). IA training creates a stable memory trace in a single trial and causes substantial changes in gene expression in CA1, a site of robust synaptic plasticity (Whitlock et al., 2006). Training allowed mice to escape from an illuminated field into a dark chamber where a short electric footshock was delivered, and subsequent context association was allowed for 15 s. Hippocampi of trained and control animals were biochemically analyzed for pS9-GSK3ß, which was increased at 30 and 180 min post-IA-training, relative to naive mice (Fig. 1A). Total GSK3ß protein levels were not significantly altered by training (Fig. 1A, Fig. S1).

We extended this finding to another type of hippocampus dependent memory, i.e. the object recognition test (ORT). Training consisted of 10 min of object exploration, 24 h after the open field exploration. Hippocampi of naive mice, of mice handled without object exposure and of mice trained with an object were biochemically analyzed 30 and 180 min post-training for pS9-GSK3ß and total GSK3ß concentrations. Levels of pS9-GSK3 were significantly increased in hippocampus of trained mice at 180 min post-training relative to naive mice (Fig. 1B), while total GSK3ß concentrations were unchanged.

The data demonstrate that training of mice in hippocampus dependent tasks increased the phosphorylation of hippocampal GSK3ß-S9, a direct biochemical measure of its inhibition.

Inhibitory phosphorylation of GSK3ß is essential for long term memory formation

To further define whether inhibitory phosphorylation of GSK3ß is essential to cognition, we analyzed mice that express the phosphorylation defective mutant, GSK3ß[S9A] (Spittaels et al., 2000) for behavior and in a battery of cognitive tests, i.e. inhibitory avoidance, object recognition, conditioned taste aversion and cued and contextual fear conditioning.

First, explorative behavior, anxiety and locomotion of GSK3ß[S9A] mice were measured during a short time-span (10 min) as in cognitive tests. Open field and light–dark tests revealed no significant differences in explorative, locomotive or anxiety related behavior by following parameters: time spent in different zones, travel speed and number of entries in different zones (Fig. S2 and results not shown).

We next assessed cognitive performance in inhibitory avoidance, known to be hippocampus dependent (Isaacson and Wickelgren, 1962). Inhibitory avoidance memory with a mild stimulation protocol revealed no differences in the retention index between non-transgenic and GSK3ß[S9A] mice. However, non-transgenic mice

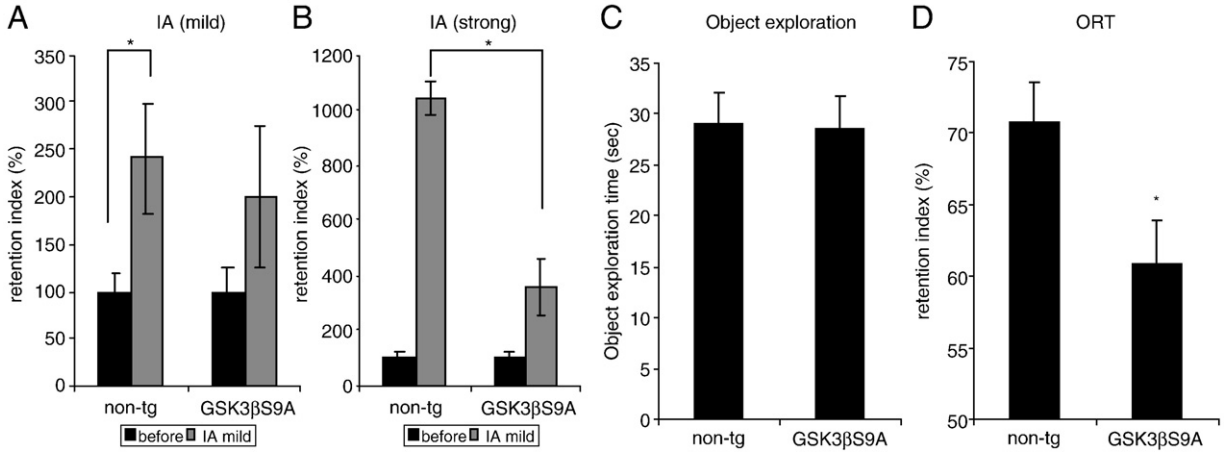

Fig. 2. Phosphorylation defective GSK3ß[S9A] impaired long term memory in hippocampus dependent tasks. (A, B) Inhibitory avoidance (IA) task, performed with mild (A) and strong (B) stimulation, demonstrated a marked deficiency in IA memory in GSK3ß[S9A] mice, measured as the relative increase in latency to enter the dark compartment associated with footshock delivery 24 h post-training (retention index). Training using a mild stimulation only induced significant inhibitory avoidance memory in non-transgenic mice (*$p<0.05$) and not in GSK3ß[S9A] mice ($n=8$; *$p<0.05$). IA memory using a strong stimulation protocol was significantly impaired in the GSK3ß[S9A] mice compared to wild-type mice ($n=8$; *$p<0.05$). (C, D) Retention indices measured 4 h after training in the novel object recognition task were significantly lower in GSK3ß[S9A] mice than in non-transgenic mice ($n=16$; *$p<0.05$) (D). Exploratory behavior was not affected by the genotype (C). Retention indices are calculated as $100*(t_{new}/(t_{old}+t_{new}))$.

displayed significantly increased latency to enter the dark compartment 24 h post-training, indicating significant memory formation. In contrast, GSK3ß[S9A] mice failed to display significant differences in latency 24 h post-training (Fig. 2A) indicating the absence of formation of inhibitory avoidance memory. We then assessed cognitive functioning in the IA task using stronger stimulation. The latency to enter the dark compartment at 24 h post-training was increased approximately 10-fold in non-transgenic mice, but only 3.5 fold in GSK3ß[S9A] mice (Fig. 2B). The GSK3ß[S9A] mice thereby demonstrated a dramatic impairment of IA memory compared to non-transgenic littermates.

These observations were subsequently extended to a different hippocampus dependent cognitive task based on object recognition. The retention index, measured 4 h after learning, revealed a significant impairment in GSK3ß[S9A] mice compared to non-transgenic, age and sex-matched littermates (Fig. 2D). No differences were observed in explorative behavior (Fig. 2C), nor in retention 1 h after learning (results not shown). These results demonstrate a significant impairment in long term object recognition memory in GSK3ß[S9A] mice.

Additional cognitive analysis by cued and contextual fear conditioning tasks and by conditioned taste aversion, revealed no significant differences of GSK3ß[S9A] mice relative to non-transgenic littermates (Fig. S3). This indicated that mechanisms involved in these tasks are not affected by GSK3ß, or alternatively that the learning stimuli of the tests were inadequate to affect GSK3ß dependent pathways.

The combined results of the comprehensive set of behavioral experiments demonstrate that GSK3ß[S9A] mice suffer a selective impairment of cognitive functions and imply a role for GSK3ß in long term memory.

It must be noted that GSK3ß[S9A] mice did not display neurodegeneration nor inflammation, assessed by various markers, including staining for NeuN, FluoroJadeB, GFAP and CD45 (Fig. S4), indicating that cognitive defects were not caused by neurodegeneration or inflammation.

Inhibitory phosphorylation of GSK3ß bidirectionally modulates synaptic plasticity, impairing L-LTP and facilitating LTD

To further corroborate the role for GSK3ß in long term memory, we analyzed GSK3ß[S9A] mice and non-transgenic littermates for ex vivo synaptic plasticity, generally considered as the cellular correlate of learning and memory.

Induction of LTP by weak stimulation with a single train as used for early LTP (E-LTP) induced very similar potentiation in non-transgenic and GSK3ß[S9A] transgenic mice (Fig. 3B). L-LTP, defined by its dependency on gene transcription and protein translation, was induced by a strong stimulation protocol, i.e. 4 trains interspaced by 5 min, and measured 3 h after induction. L-LTP was significantly impaired in GSK3ß[S9A] transgenic mice compared to non-transgenic mice (Fig. 3A).

Basal synaptic transmission and paired pulse facilitation, parameters of presynaptic functioning, were not significantly affected in GSK3ß[S9A] mice (Fig. S5). Finally, induction of LTD by low frequency stimulation of Schaffer collaterals, i.e. 900 stimuli at 1 Hz, resulted in significant higher facilitation of LTD in hippocampal slices from GSK3ß[S9A] mice, relative to non-transgenic mice (Fig. 3C).

The data indicate that GSK3ß is crucially involved in regulating synaptic plasticity, by demonstrating inhibited L-LTP and facilitated LTD in GSK3ß[S9A] mice. The synaptic defects correlated spatially-hippocampus- and temporally with the defects in cognition, in the absence of neuronal loss or inflammation.

GSK3ß modulates transcriptional regulation involved in late-LTP

To pinpoint the molecular actions involved in inhibition of L-LTP in the GSK3ß[S9A] transgenic mice, the effects of GSK3ß[S9A] expression were compared with the effects of well-characterized inhibitors of protein translation and transcription, both demonstrated to be essential for L-LTP.

Anisomycin, an inhibitor of translation, rapidly impaired L-LTP in brain sections from non-transgenic mice, which was significantly different from the impairment observed in sections from GSK3ß[S9A] mice (results not shown). Addition of actinomycin, a well-known inhibitor of L-LTP by inhibition of transcription, significantly impaired L-LTP in non-transgenic mice at 200 min following tetanic stimulation, a very similar time-point as observed in GSK3ß[S9A] mice (Fig. 4A). L-LTP in GSK3ß[S9A] mice was not significantly different from non-transgenic mice treated with actinomycin, suggesting modulation of L-LTP by GSK3ß by transcriptional regulation.

Finally, to further elaborate the impact of GSK3ß[S9A] expression on transcriptional regulation underlying L-LTP, CREB dependent

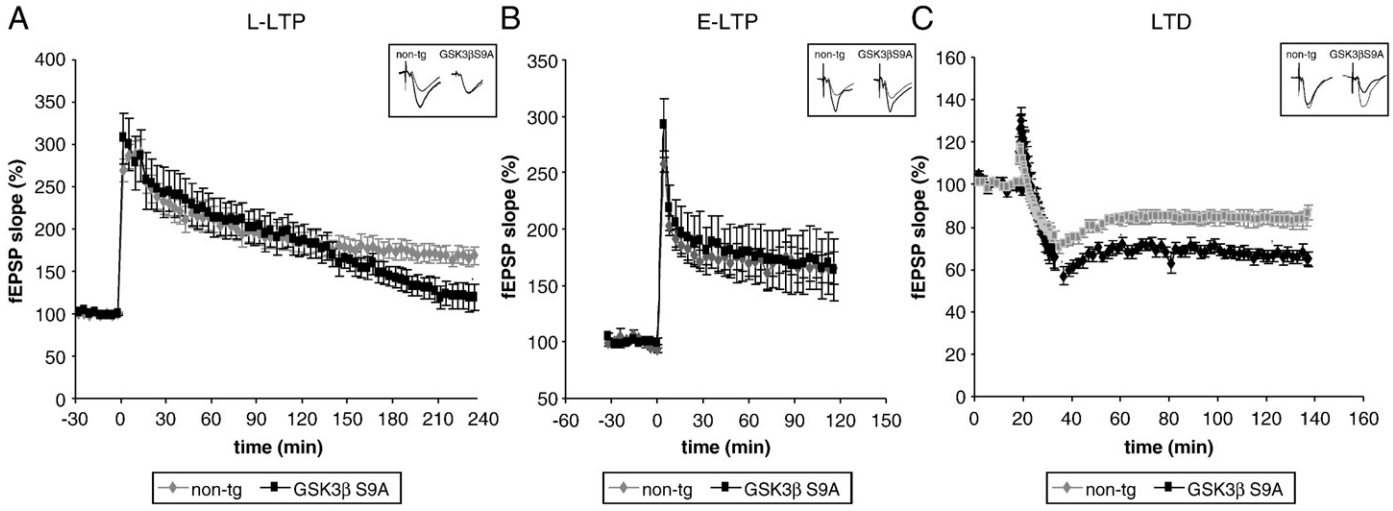

Fig. 3. Impaired L-LTP and facilitated LTD in GSK3ß[S9A] mice underlines the role of GSK3 in bidirectional modulation of synaptic plasticity. (A) L-LTP, induced by 4 trains of 100 Hz with 5 min intervals, was significantly impaired in GSK3ß[S9A] mice at 180 min and 240 min after induction ($n=7$; $^*p<0.05$) (B) Weak stimulation, i.e. 1 train of 100 Hz, used for induction of E-LTP, induced similar responses in GSK3ß[S9A] mice and non-transgenic mice ($n=6$). (C) Low frequency stimulation (900 pulses, 1 Hz) elicited weak LTD in non-transgenic mice that was significantly facilitated in hippocampal slices of GSK3ß[S9A] mice ($n=12$; $^*p<0.05$).

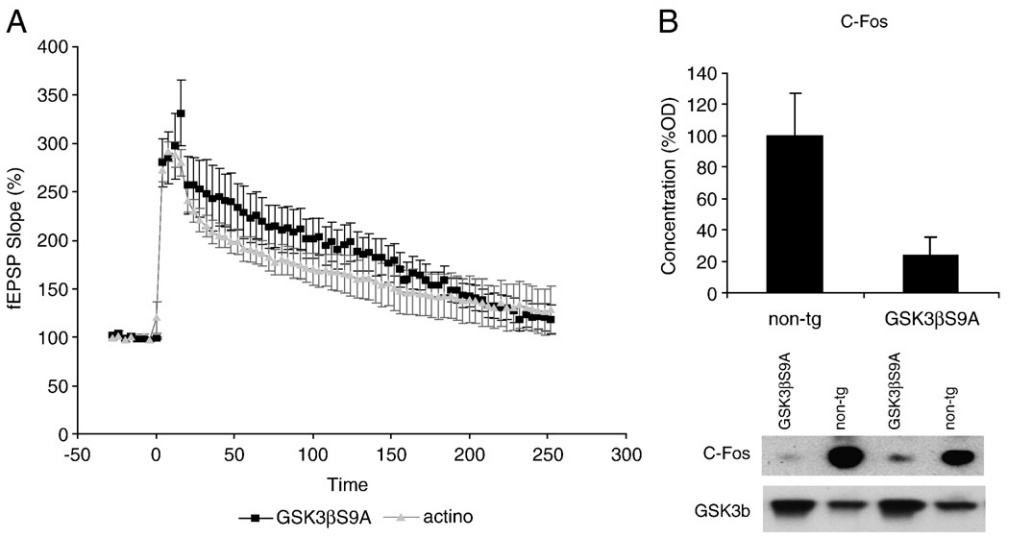

Fig. 4. Implication of deregulated transcriptional activation by GSK3ß[S9A]. (A) Application of actinomycin (25 μM) in non-transgenic mice, a well-characterized inhibitor of transcription, similarly affects Late-LTP as expression of phosphorylation defective GSK3ß[S9A] ($n=4$, $n=6$). (B) Expression of *c-Fos*, an immediate early gene and downstream target of CREB, is significantly decreased in brain of GSK3ß[S9A] mice compared to non-transgenic mice ($n=5$, $p<0.05$). A representative Western blot is presented in the lower panel.

transcriptional activation was analyzed by measuring *c-Fos*, an immediate early gene involved in long term memory formation and known target of CREB. Western blotting revealed a significant decrease in the concentration of c-Fos in the brain of GSK3ß[S9A] mice (Fig. 4B).

Discussion

In this work we demonstrate a crucial role of GSK3ß in long term memory by inhibitory phosphorylation on Serine-9. To identify the critical involvement of a signaling pathway in long term memory, two criteria need to be fulfilled. First, behavioral training that induces memory should activate the biochemical cascade. Second, blockade within this cascade should interrupt the response. This is unequivocally demonstrated by the fact that hippocampal GSK3ß is phosphorylated at Serine-9 following cognitive testing in 2 different hippocampus dependent tasks. Conversely, we demonstrate that blocking phosphorylation of GSK3ß at Serine-9 in vivo, impaired long term memory in inhibitory avoidance and object recognition tasks in vivo, i.e. in mice with postnatal neuronal expression of the constitutively active GSK3ß[S9A]. Moreover, further analysis revealed impairment of L-LTP, while LTD was facilitated in mice expressing GSK3ß[S9A]. Our data demonstrate a role for GSK3ß in long term memory by bidirectional modulation of synaptic plasticity.

The current study demonstrates the induction of phosphorylation of GSK3ß on S9 following cognitive training in the object recognition test and the passive inhibitory avoidance, one of the most robust cognitive paradigms linked to induction of LTP (Whitlock et al., 2006). Inhibition of GSK3ß by phosphorylation on S9 by the Akt kinase is part of a well-documented signaling cascade (Cross et al., 1995). Interestingly, activation of Akt by PI3k occurs following learning and induction of LTP, while blockade of PI3k signaling prevents learning and LTP (Lin et al., 2001, Sanna et al., 2002, Liu et al., 2003). The role for GSK3ß in learning and memory demonstrated here, could well depend upstream on regulation by PI3k, with GSK3ß as the downstream effector, but other upstream signals are not excluded.

Our data are in line with the only report on the effect of GSK3ß expression on memory in vivo, i.e. impaired spatial memory in the water-maze paradigm in transgenic mice conditionally overexpressing GSK3ß (Lucas et al., 2001; Hernández et al., 2002). Importantly, those conditional GSK3ß mice express the kinase to much higher levels than the GSK3ß[S9A] mice used in the current study, resulting in hippocampal neurodegeneration with pronounced gliosis (Lucas et al., 2001). Cognitive deficits might be due to GSK3ß expression or alternatively to secondary effects like neuronal loss, neurodegenerative processes or inflammation. The GSK3ß[S9A] used in the current study do not display signs of neurodegeneration nor inflammation even at old age.

The role of GSK3ß in synaptic plasticity is demonstrated by the impairment of L-LTP, with unaffected E-LTP, associated moreover with facilitated LTD in the mice expressing constitutively active GSK3ß. Most recently, GSK3ß was proposed to help switch off LTD following induction of LTP, based on pharmacological inhibition of GSK3 (Peineau et al., 2007, 2008). Those data elegantly support the mechanism that is invoked to protect synapses from additional NMDA-receptor dependent input, until the previous information is either consolidated or erased. Independent reports provided indications that GSK3ß does affect LTP (Hooper et al., 2007, Zhu et al., 2007). Our current study significantly extends the data specifically to bidirectional modulation of synaptic plasticity by GSK3ß, by the demonstrated inhibition of L-LTP and the facilitation of LTD. The same previous reports demonstrated the role for GSK3ß in the induction of LTP using weak stimulation, while we did not. This apparent discrepancy is thought to arise from the fact that the level of activation and inhibition of GSK3ß will be critical in its modulation of synaptic plasticity. Conversely, the absence of an effect on E-LTP in our mice allowed us to demonstrate the role of GSK3ß on L-LTP and its behavioral correlate long term memory. Interestingly, the different outcomes in models pending on the level of activation of GSK3, may relate to different clinical phenotypes and effects on cognitive phenotypes observed in neurological disorders.

Our analysis of molecular mechanisms underlying the impaired L-LTP points to transcriptional regulation, based on the similarity of the effect of actinomycin, the well-characterized inhibitor of transcription, and the effect of GSK3ß[S9A] expression. Furthermore, expression of the immediate early gene *c-Fos* was significantly decreased in GSK3ß[S9A] mice. CREB dependent transcription and particularly induction of immediate early genes, including *c-Fos* and *zif268* are crucial to L-LTP and long term memory (Fleischmann et al., 2003, Cole et al., 1989, Jones et al., 2001). Increased phosphorylation of CREB on S129 by GSK3ß has been demonstrated to decrease CREB activity (Bullock and Habener, 1998; Gonzalez and Montminy, 1989) and reduce expression of immediate early genes in vitro (Tullai et al., 2007). In view of the essential role of the induction of immediate

early genes in long term memory, and the similarities between actinomycin and GSK3ß[S9A] expression as well as decreased expression of c-Fos in GSK3ß[S9A] mice, our data point to regulation of immediate early genes as crucial molecular mechanism involved in GSK3ß dependent regulation of L-LTP and memory.

Cognition is considered an important emerging therapeutic target in neuropsychiatric conditions. Converging evidence relates cognitive impairment to functional outcomes in neuropsychiatric disorders (Green, 2006; Burdick et al., 2007; Martinez-Aran et al., 2007) and supports cognitive impairment as a core feature of neuropsychiatric illnesses.

Worth noting is that the GSK3ß[S9A] mice used in this study represent endophenotypes of bipolar disorder, particularly modeling the manic phase (Prickaerts et al., 2006). Evidence is emerging that cognitive impairment is a core feature of bipolar disorder, while cognitive deficits correlate with and predict functional outcomes (Green, 2006; Powell and Miyakawa, 2006; Einat and Manji, 2006). The GSK3ß[S9A] mice represent an endophenotypical model with pronounced defective long term memory. Our current data thereby point to GSK3ß as a therapeutic target for cognitive dysfunction in these disorders.

The critical involvement of GSK3ß in cognitive functions furthermore underlines its potential as a therapeutic target in early cognitive dysfunction in Alzheimer's disease and other dementias. In view of the implication of GSK3 in Tau phosphorylation (Spittaels et al., 2000) and its essential role in the communication between amyloid and Tau (Terwel et al., 2008), and even in APP-processing, GSK3 represents an interesting therapeutic target. Unwanted side-effects must be carefully considered and countered by further studies in pre-clinical models already available or being generated, although only clinical studies will decide.

In conclusion, this study demonstrates that inactivation of GSK3ß by phosphorylation of Serine-9 is intimately involved in long term memory. We believe that these findings are not only fundamentally relevant, but also translationally important in view of the role of GSK3 in a variety of neurological disorders associated with cognitive dysfunction, e.g. bipolar disorder, schizophrenia, mania, depression, anxiety disorders and including Alzheimer's disease as the most frequent disorder.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2009.04.003.

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