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Keywords

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Comments

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In Vivo Regulation of Glycogen Synthase Kinase-3 β (GSK3 β) by Serotonergic Activity in Mouse Brain

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Abstract

The goal of this study was to determine if serotonergic activity, which is impaired in depression, regulates the phosphorylation of glycogen synthase kinase-3 β (GSK3 β) in mouse brain *in vivo*. GSK3 β is inhibited by phosphorylation on serine-9 and is a target of the mood stabilizer lithium. Following administration to mice of *d*-fenfluramine to stimulate serotonin (5HT) release and reduce its reuptake, and clorgyline to inhibit 5HT catabolism, levels of phospho-Ser9-GSK3 β were 300–400% of control levels in the prefrontal cortex, hippocampus, and striatum. Treatment with monoamine reuptake inhibitors fluoxetine and imipramine also increased the level of phospho-Ser9-GSK3 β . Using receptor selective agonists and antagonists, 5HT1A receptors were found to mediate increases, and 5HT2 receptors decreases, in phospho-Ser9-GSK3 β levels. This indicates that serotonergic regulation of the phosphorylation of GSK3 β is achieved by a balance between the opposing actions of these 5HT receptor subtypes. These findings demonstrate for the first time that serotonergic activity regulates the phosphorylation of GSK3 β and show that this regulation occurs in mammalian brain *in vivo*. These results raise the possibility that impaired inhibitory control of GSK3 β may occur in conditions where serotonergic activity is dysregulated, such as in mood disorders.

Keywords

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INTRODUCTION

Glycogen synthase kinase-3 (GSK3) has recently become recognized as a broadly influential enzyme that modulates many aspects of neuronal function (Grimes and Jope, 2001). GSK3 is now known to exert profound influences on cellular architecture and plasticity, to regulate multiple transcription factors and consequently the expression of many genes, and to contribute to the regulation of apoptosis, and thus cell survival (Frame and Cohen, 2001; Grimes and Jope, 2001). In accordance with these many important actions, the activity of GSK3 is tightly regulated. The phosphorylation state of GSK3 is the most well-characterized mechanism that regulates its activity, although regulation of its subcellular distribution and interactions with GSK3-binding proteins also contribute to controlling its actions (Jope and Johnson, 2004). Both of the two known isoforms of GSK3 are inhibited by phosphorylation of an N-terminal serine, which involves serine-9 in GSK3 β and serine-21 in GSK3 α . This site can be phosphorylated by several different kinases, including Akt (also known as protein kinase B) (Cross *et al*, 1995), protein kinase A (Fang *et al*, 2000; Li *et al*, 2000), protein kinase C (Goode

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et al., 1992), and others, indicating that many signaling cascades converge on GSK3 to regulate its activity. Impairments of this inhibitory control of GSK3 can result in abnormally high GSK3 activity, a condition that can have detrimental effects on neural plasticity, structure, and survival (Jope and Johnson, 2004). In opposition to the inhibitory N-terminal serine phosphorylation, tyrosine phosphorylation (tyrosine-216 in GSK3 β and tyrosine-279 in GSK3 α) increases the activity of GSK3, but this appears to contribute a lesser regulatory effect than serine phosphorylation (Frame and Cohen, 2001) and the mechanisms regulating tyrosine phosphorylation remain unclear. Recently, GSK3 β (the isoform most often investigated) has received much attention from investigators studying mood disorders because the mood stabilizer lithium was found to be a direct inhibitor of GSK3 β , a finding that raised the possibility that GSK3 β may not be adequately controlled in mood disorders (Klein and Melton, 1996; Phiel and Klein, 2001; Jope, 2003).

Impaired monoaminergic neurotransmission has long been linked to depression. This is indicated in part because the majority of antidepressant drugs increase levels of the monoamines serotonin (5HT) and/or norepinephrine, and drugs selectively affecting 5HT are especially in wide use. Thus, pharmacological augmentation of 5HT with monoamine oxidase (MAO) inhibitors, tricyclic antidepressants, or selective serotonin reuptake inhibitors (SSRIs), can be therapeutic for depression (Duman *et al.*, 1997). Furthermore, dysregulation of 5HT neurotransmission in major depression is substantiated by results using a wide variety of experimental approaches, including studies of 5HT receptors and transporters (Middlemiss *et al.*, 2002; Stockmeier, 2003), brain imaging and post-mortem studies (Drevets *et al.*, 1999; Ichimiya *et al.*, 2002; Stockmeier, 2003), 5HT metabolism (Owens and Nemeroff, 1994), and neuroendocrine actions (Yatham and Steiner, 1993). Regardless of whether mood disorders involve impairments in 5HT synthesis, release, or receptor-stimulated actions, any of these can result in deficient downstream signaling activity. Therefore, clarifying the signals generated by stimulation of 5HT receptors is an important goal for understanding the neuropathological underpinnings of mood disorders. Considering the potential importance of regulating GSK3 β in mood disorders suggested by the action of lithium, the present study was undertaken to determine if serotonergic activity regulates the phosphorylation of GSK3 β . For this purpose, the *in vivo* phosphorylation of GSK3 β was examined in three regions of mouse brain that are known to express serotonergic receptors, the prefrontal cortex, hippocampus, and striatum, following administration of agents that alter serotonergic activity.

MATERIALS AND METHODS

Animals and Treatments

Adult, male C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) were treated with the following agents by intraperitoneal injections: *d*-fenfluramine (40 mg/kg body weight), clorgyline (2 mg/kg), 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT; 0.3–10 mg/kg), WAY100635 (1 mg/kg), LY53857 (0.3–5 mg/kg), 2,5-dimethoxy-4-iodoprenyl-2-aminopropane (DOI; 20 mg/kg), haloperidol (0.2 mg/kg), imipramine (30 mg/kg; all obtained from Sigma), and fluoxetine (20 mg/kg; obtained from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program). For intraperitoneal injection of mice, all these drugs were dissolved in saline, and saline was used as vehicle controls.

Tissue Preparation

Mice were anesthetized with carbon dioxide for 15 s, decapitated, and brain regions were rapidly dissected in ice-cold saline. Brain regions were homogenized in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin, 0.1 mM β -glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, and 100 nM okadaic acid. The lysates

were centrifuged at 20 800 g for 10 min. Protein concentrations in the supernatants were determined using the Bradford protein assay (Bradford, 1976).

Immunoblotting

Samples were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 min. Proteins were resolved in 10% SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with antibodies to phospho-Ser9-GSK3 β and total GSK3 β (Cell Signaling Technology, Beverly, MA). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse, or goat anti-rabbit IgG, followed by detection with enhanced chemiluminescence, and the protein bands were quantitated with a densitometer.

RESULTS

Serotonergic Activity Regulates GSK3 β in Mouse Brain Regions *In Vivo*

To test if serotonergic signaling activity regulates the phosphorylation of GSK3 β in mouse brain *in vivo*, *d*-fenfluramine was administered to mice to stimulate 5HT release and reduce its reuptake, and 5HT catabolism was inhibited by administration of the MAO inhibitor clorgyline. Treatment with *d*-fenfluramine (40 mg/kg, 1 h) alone caused a modest increase in the levels of phospho-Ser9-GSK3 β in mouse prefrontal cortex, hippocampus, and striatum (Figure 1a). While treatment with clorgyline alone did not change phospho-Ser9-GSK3 β levels, pretreatment with clorgyline (2 mg/kg, 3 h prior to *d*-fenfluramine) enhanced the increases in phospho-Ser9-GSK3 β levels induced by *d*-fenfluramine treatment in all three brain regions. Following combined treatment with clorgyline plus *d*-fenfluramine these increases amounted to 410 \pm 107% of the control level in the prefrontal cortex ($n=11$; $p<0.01$), 425 \pm 109% in the hippocampus ($p<0.01$), and 313 \pm 63% in the striatum ($p<0.05$) (Table 1). These results reveal for the first time that serotonergic signaling activity regulates the phosphorylation of GSK3 β .

The time dependence of *d*-fenfluramine-induced increases in phospho-Ser9-GSK3 β was measured in clorgyline-pretreated mice. Administration of *d*-fenfluramine rapidly and robustly increased the levels of phospho-Ser9-GSK3 β in the prefrontal cortex, hippocampus, and striatum, while the total level of GSK3 β remained unaltered (Figures 1b and c). Maximal increases in phospho-Ser9-GSK3 β were evident in all three brain regions between 0.5 and 1 h after treatment with *d*-fenfluramine, followed by a gradual decline towards control levels over the next several hours.

To test if inhibition of monoamine reuptake alone was sufficient to modulate the phosphorylation of GSK3, measurements were made in prefrontal cortex 1 h following administration of fluoxetine (20 mg/kg), an SSRI, or imipramine (30 mg/kg), a tricyclic antidepressant that inhibits the reuptake of both 5HT and norepinephrine (Figure 2). Both treatments caused significant increases in the level of phospho-Ser9-GSK3 β , which reached 229 \pm 39% of the control level ($n=10$, $p<0.05$) after fluoxetine treatment and 268 \pm 47% of the control level ($n=4$, $p<0.05$) after imipramine treatment, whereas the total levels of GSK3 β were not changed. In contrast to fluoxetine and imipramine, administration of the antipsychotic haloperidol (0.2 mg/kg) had no effect on the level of phospho-Ser9-GSK3 β .

5HT1A Receptor Activation Increases Phospho-Ser9-GSK3 β

5HT1A receptor-selective agents were used to begin to investigate the 5HT receptor subtypes that mediate the *in vivo* 5HT-induced increase in phospho-Ser9-GSK3 β . Administration of the 5HT1A receptor antagonist WAY100635 (1 mg/kg; 2 h) alone had no significant effect on the level of phospho-Ser9-GSK3 β (Figure 3a, Table 1). However, pretreatment with WAY100635 greatly attenuated the increase in phospho-Ser9-GSK3 β levels caused by administration of clorgyline plus *d*-fenfluramine in prefrontal cortex, hippocampus, and striatum (Figures 3a and

b). Conversely, treatment with the 5HT1A receptor agonist 8-OH-DPAT (10 mg/kg; 1 h) alone significantly increased the level of phospho-Ser9-GSK3 β in all three brain regions (Figure 3a). These increases amounted to 240 \pm 55% of the control level in the prefrontal cortex (p <0.05; n =7), 239 \pm 35% in the hippocampus (p <0.05), and 362 \pm 94% in the striatum (p <0.05) (Table 1). Examination of the dose-response to 8-OH-DPAT revealed that a dose as low as 0.3 mg/kg (hippocampus) or 1 mg/kg (prefrontal cortex and striatum) caused increased phospho-Ser9-GSK3 β , while the total level of GSK3 β was unaltered (Figure 3c). These results indicate that activation of 5HT1A receptors increases phospho-Ser9-GSK3 β in mouse brain *in vivo*.

Phospho-Ser9-GSK3 β is Increased by Blocking 5HT2 Receptors

To examine if regulation of 5HT2 receptors influences the phosphorylation of GSK3 β , we utilized the 5HT2 receptor agonist DOI and antagonist LY53857. Whereas activation of 5HT2 receptors with DOI (20 mg/kg; 1 h) did not change phosphorylation of GSK3 β (Figure 4a, Table 1), administration of LY53857 (5 mg/kg; 2 h) alone caused a large increase in phospho-Ser9-GSK3 β in prefrontal cortex (346 \pm 97% of control level, n =7, p =0.055), hippocampus (193 \pm 30%; p <0.05), and striatum (378 \pm 60; p <0.01) without changing the total level of GSK3 β (Figure 4a, Table 1). The duration of the increased phospho-Ser9-GSK3 β following blockade of 5HT2 receptors was examined by making measurements 1, 2, 4, 6, and 24 h after a single administration of LY53857 (5 mg/kg) (Figure 4b). Treatment with LY53857 caused a rapid and prolonged increase in phospho-Ser9-GSK3 β in all three brain regions followed by a return to control levels after 24 h. Examination of the dose-response to LY53857 demonstrated that increased phosphorylation of GSK3 β was dose dependent with the effects more prominent in the prefrontal cortex and striatum than in the hippocampus (Figure 4c). The lack of an effect of DOI and the increases in phospho-Ser9-GSK3 β caused by LY53857 indicate that endogenous activity of 5HT2 receptors contributes to the maintenance of dephosphorylated GSK3 β and that maximal signaling of 5HT2 receptors to reduce phospho-Ser9-GSK3 β was achieved by endogenous 5HT in the absence of further stimulation.

These findings were used to examine the effect of blocking 5HT2 receptors, which normally mediate dephosphorylation of GSK3 β , on the phosphorylation of GSK3 β caused by stimulating serotonergic activity. Pretreatment with LY53857 to block 5HT2 receptors followed by administration of clorgyline plus *d*-fenfluramine resulted in a very large increase in phospho-Ser9-GSK3 β (Figure 4d). This supports the conclusion that activation of 5HT2 receptors following administration of clorgyline plus *d*-fenfluramine was inhibitory towards the phosphorylation of GSK3 β , an effect opposite to that of 5HT1A receptors. To further examine these opposing actions of the 5HT1A and 5HT2 receptors, 5HT1A receptors were stimulated by administration of 8-OH-DPAT and 5HT2 receptors were blocked by treatment with LY53857. These results showed that blocking 5HT2 receptors allowed a greater 8-OH-DPAT-induced increase in phospho-Ser9-GSK3 β than was caused by administration of the 5HT1A agonist alone (Figure 4e). Taken together, these results demonstrate that serotonergic regulation of phospho-Ser9-GSK3 β is achieved by a balance between the opposing actions of 5HT1A and 5HT2 receptors (Figure 5).

DISCUSSION

The results of this study show that enhanced serotonergic activity in several regions of mouse brain increases the inhibitory Ser9-phosphorylation of GSK3 β . It is of interest that this effect also is caused *in vivo* by a therapeutically relevant concentration of lithium (De Sarno *et al*, 2002). Examination of receptor subtypes involved in the serotonergic regulation of phospho-Ser9-GSK3 β revealed that stimulation of 5HT1A receptors causes phosphorylation of GSK3 β , whereas 5HT2 receptor stimulation mediates dephosphorylation of GSK3 β . Thus, the balance between the activities of these 5HT receptors can modulate GSK3 β *in vivo*.

Previous studies have provided evidence that 5HT1A receptors are deficient in major depressive disorder, although this remains a subject of intense investigation (reviewed in Stockmeier, 2003). For example, positron emission tomography measurements of brain 5HT1A receptors identified widespread reductions associated with major depressive disorder (Drevets *et al*, 1999; Sargent *et al*, 2000). Furthermore, a recent report showed that 5HT1A receptor-coupled activation of signal transduction systems was reduced in post-mortem brain samples from depressed suicide victims compared with matched controls (Hsiung *et al*, 2003). Also relevant was their finding that Akt activity is substantially lower in post-mortem brain samples from depressed suicide victims than in control samples (Hsiung *et al*, 2003). This is of interest because Akt normally causes inhibitory Ser9-phosphorylation of GSK3 β (Cross *et al*, 1995), so its impairment also could contribute to abnormally active GSK3 β . Our finding that stimulation of 5HT1A causes Ser9-phosphorylation (inactivation) of GSK3 β , raises the possibility that low 5HT1A activation in depression may result in deficient inhibitory control of GSK3 β through impaired phosphorylation, resulting in abnormally active GSK3 β . However, this postulate is difficult to address directly because we previously found that phospho-Ser9-GSK3 β is dephosphorylated post mortem and large variations were observed among post-mortem samples from human subjects (Lesort *et al*, 1999, and unpublished observations). Only with the development of new methodologies will experiments be able to accurately measure the activity or phosphorylation state of GSK3 β in human brain samples.

Opposite to the downregulated 5HT1A receptors, there is substantial evidence that 5HT2 receptors are upregulated in depression (reviewed in Stockmeier, 2003). The present study found that stimulation of 5HT2 receptors supports dephosphorylation (activation) of GSK3 β . Thus, it can be surmised that upregulated 5HT2 receptors in depression may be associated with abnormal increases in GSK3 β activity in depression. The results also showed that endogenous serotonergic activity, in the absence of any stimulant, acts predominantly through 5HT2 receptors to keep GSK3 β dephosphorylated because treatment with the 5HT2 antagonist LY53857 increased phospho-Ser9-GSK3 β , whereas treatment with the 5HT1A receptor antagonist WAY100635 had no effect without additional serotonergic stimulation. The basal 5HT2 receptor-mediated dephosphorylation of GSK3 β was maximal, because it was unaltered by administration of the 5HT2 agonist DOI. In contrast, the 5HT1A receptor effect could be activated further by stimulation, because administration of the 5HT1A agonist 8-OH-DPAT increased phospho-Ser9-GSK3 β , and serotonergic stimulation (with administration of clorgyline plus *d*-fenfluramine or 5HT reuptake inhibitors) increased phospho-Ser9-GSK3 β , an increase that was greatly attenuated by the 5HT1A receptor antagonist WAY100635. Taken together, reduced signaling capacity of 5HT1A receptors which would disable inhibitory regulation of GSK3 β , and increased signaling capacity of 5HT2 receptors which activate GSK3 β , by acting together could cause a substantial increase in GSK3 β activity in major depression. It is not yet known whether these modulatory effects of serotonergic activity on GSK3 β emanate directly from 5HT receptor-coupled signaling pathways or if they reflect changes in the activities of neuronal circuits influenced by 5HT, a question that is currently under investigation.

These findings suggest the possibility that reduced serotonergic activity, as appears to occur in depression, is associated with deficient inhibitory control of GSK3 β , raising the question of how this condition would affect neuronal function. The answer to this rests with what is known about the actions of GSK3 β . This enzyme has extensive effects on neuronal function, including influences on gene expression through the regulation of at least 15 different transcription factors (Jope and Johnson, 2004). Equally important, GSK3 β extensively influences neuronal architecture, plasticity, intracellular transport mechanisms, and even survival, generally impairing these events (Grimes and Jope, 2001), so modulation of GSK3 activity can underlie structural dynamics and neural plasticity over time. This may go so far as to include neuronal survival, a relevant possibility because imaging and post-mortem studies indicate that

depression is associated with cell loss (Rajkowska, 2002) and GSK3 is well known to promote cell death (Grimes and Jope, 2001). Thus, hyperactive GSK3 β associated with dysregulated serotonergic activity could detrimentally affect neuronal structure, plasticity, and survival, and may be amenable to regulation by antidepressant treatments, which is currently under investigation.

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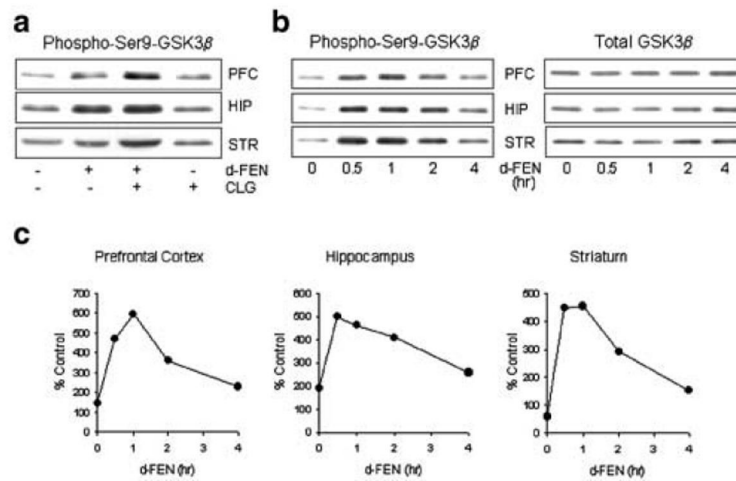


Figure 1.

Serotonergic stimulation increases phospho-Ser9-GSK3 β *in vivo*. (a) Mice were treated with *d*-fenfluramine (*d*-FEN; 40 mg/kg, 1 h), clorgyline (CLG; 2 mg/kg; 3 h prior to *d*-FEN), or CLG alone (4 h), and phospho-Ser9-GSK3 β levels were measured by immunoblot analysis in prefrontal cortex (PFC), hippocampus (HIP), and striatum (STR). (b) Representative immunoblots of phospho-Ser9-GSK3 β (left) and total levels of GSK3 β (right), and (c) quantitation of phospho-Ser9-GSK3 β levels, showing the time dependence of *d*-FEN-induced increases in phospho-Ser9-GSK3 β in CLG-pretreated mice. Values from a representative experiment are shown as the percent of control values from vehicle (saline)-treated mice, and values at 0 h were obtained in samples from mice treated with CLG alone.

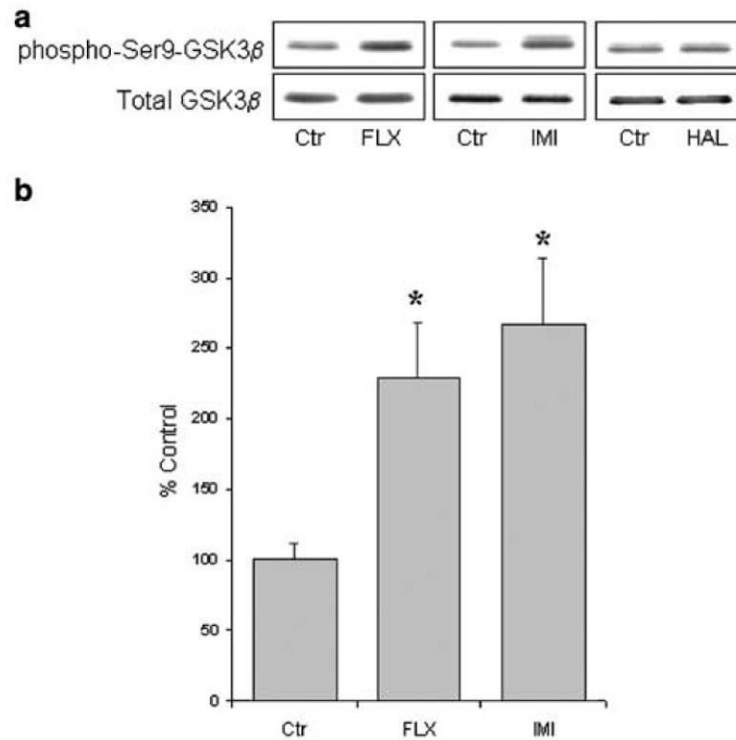


Figure 2.

Monoamine reuptake inhibitors increase phospho-Ser9-GSK3 β *in vivo*. (a) Representative immunoblots of phospho-Ser9-GSK3 β and total GSK3 β , and (b) quantitative values of phospho-Ser9-GSK3 β level (percent of saline control), in PFC 1 h after treatment with saline (Ctr), fluoxetine (FLX; 20 mg/kg; $n=10$), imipramine (IMI; 30 mg/kg; $n=4$), or haloperidol (HAL; 0.2 mg/kg). Means \pm SEM; * $p<0.05$ compared with control (saline) values (ANOVA).

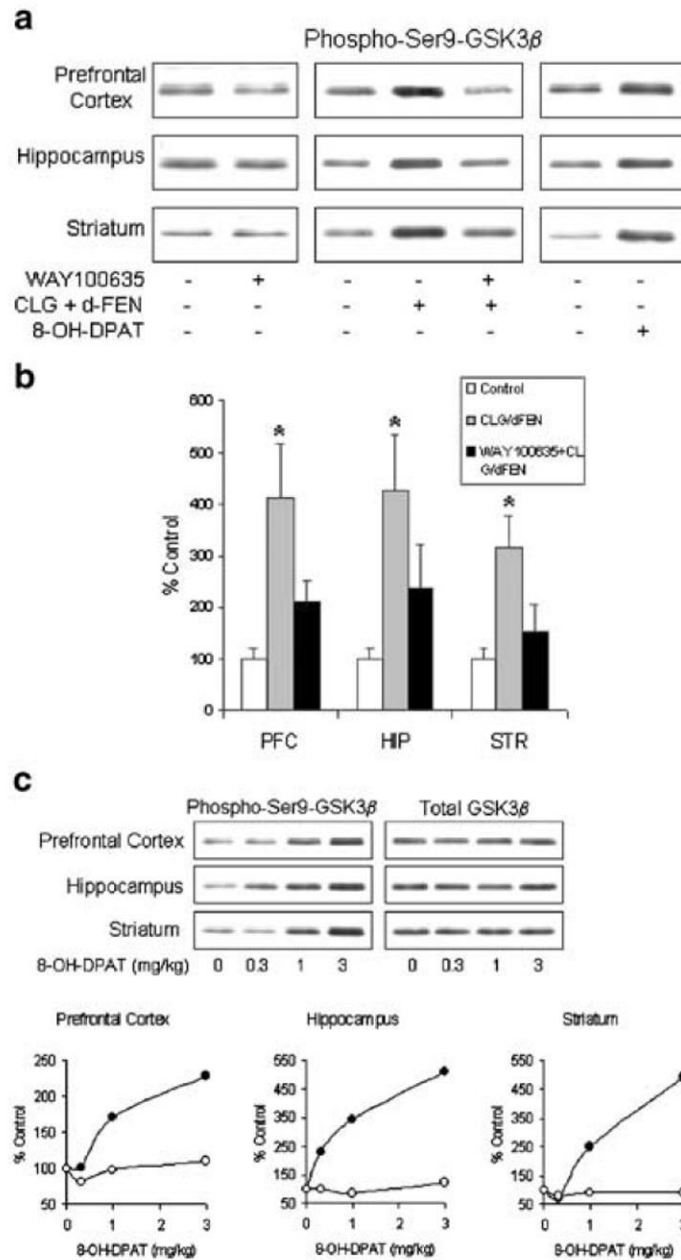
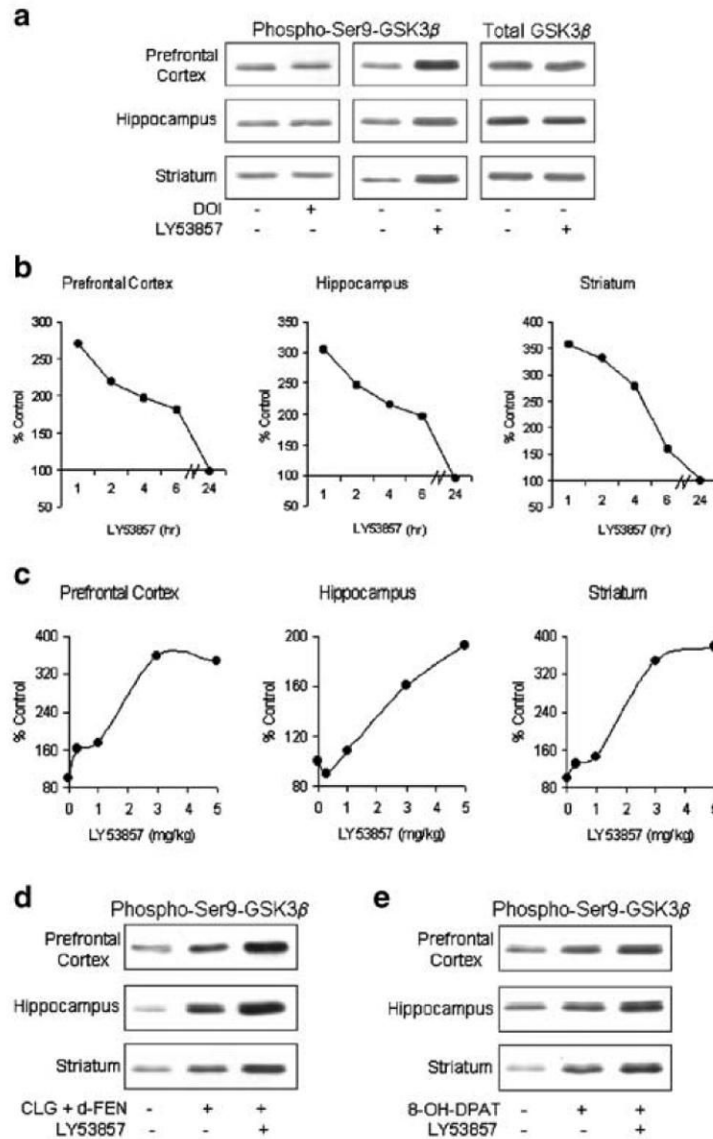


Figure 3.

Phospho-Ser9-GSK3 β is increased by stimulation of 5HT1A receptors. (a) Phospho-Ser9-GSK3 β was measured in PFC, HIP, and STR following administration of the 5HT1A receptor antagonist WAY100635 (1 mg/kg; 2 h), CLG (2 mg/kg) 3 h prior to *d*-FEN (40 mg/kg; 1 h), CLG 3 h and WAY 100635 1 h prior to *d*-FEN, or the 5HT1A receptor agonist 8-OH-DPAT (10 mg/kg; 1 h). The total level of GSK3 β also was measured in each sample and was not changed by any of these treatments (data not shown). (b) Quantitative values (given as the percent of saline controls) of the stimulation of phospho-Ser9-GSK3 β following treatment with CLG prior to *d*-FEN (shaded bars) and the attenuation of that stimulation provided by pretreatment with WAY100635 (black bars) (treatment protocols as described in (a)) in PFC, HIP, and STR. Means \pm SEM. * p <0.05 compared with control (saline) values (ANOVA). (c)

Immunoblots and quantitative values of concentration-dependent effects of treatment with 8-OH-DPAT (0, 0.3, 1.0, and 3.0 mg/kg; 1 h) on phospho-Ser9-GSK3 β (closed circles) and total GSK3 β (open circles) in PFC, HIP, and STR. Values from a representative experiment are shown as the percent of values from control (saline-treated) mice.

**Figure 4.**

Phospho-Ser9-GSK3 β is increased by blocking 5HT₂ receptors. (a) Phospho-Ser9-GSK3 β was measured by immunoblots in PFC, HIP, and STR following administration of the 5HT₂ receptor agonist DOI (20 mg/kg; 1 h) or the 5HT₂ receptor antagonist LY53857 (5 mg/kg; 2 h). Immunoblots of total GSK3 β show that it was unaltered by treatment with LY53857. (b) Quantitation of phospho-Ser9-GSK3 β levels, showing the duration of increases in phospho-Ser9-GSK3 β induced by LY53857 (5 mg/kg) in three mouse brain regions. (c) Concentration-dependent effects of treatment with LY53857 (0, 0.3, 1, 3, and 5 mg/kg; 2 h) on phospho-Ser9-GSK3 β . Values in (b) and (c) are shown as the percent of values from control (saline-treated) mice. (d) LY53857 was administered 1 h prior to treatment with *d*-FEN (40 mg/kg, 1 h) in CLG-pretreated mice. (e) 8-OH-DPAT (10 mg/kg; 1 h) was administered alone or 1 h after LY53857 (5 mg/kg; 2 h).

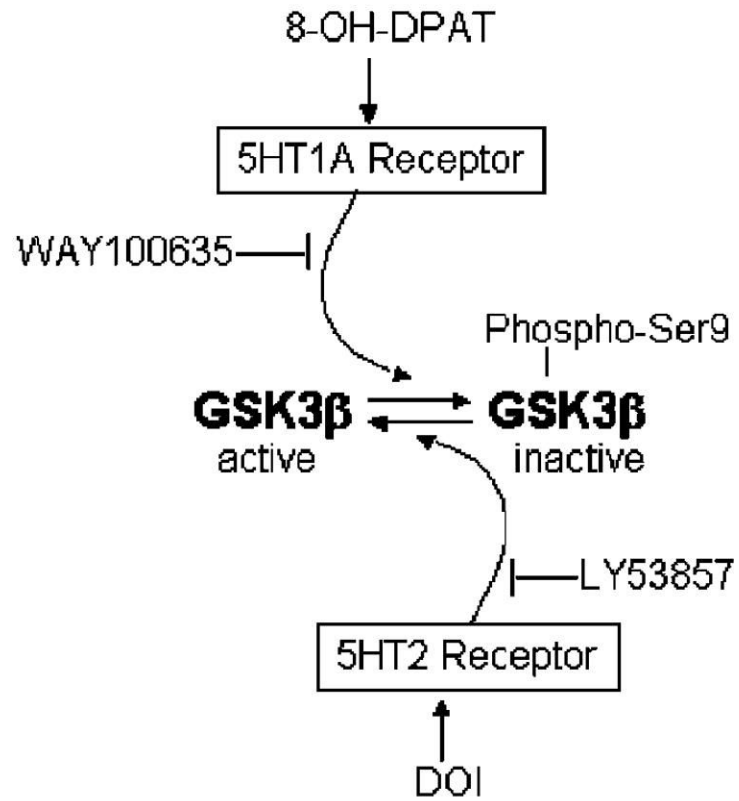


Figure 5. Schematic depiction of the regulation of GSK3 β activity by 5HT1A and 5HT2 receptors.

Table 1

Effects of Serotonergic Agents on Phospho-Ser9-GSK3 β

Drug	Phospho-Ser9-GSK3 β (% Control)					
	Prefrontal cortex		Hippocampus		Striatum	
	Ave \pm SEM	n	Ave \pm SEM	n	Ave \pm SEM	n
Clorgyline +d-fenfluramine	410 \pm 107 ^{**}	11	425 \pm 109 ^{**}	10	313 \pm 63 [*]	11
WAY100635	120 \pm 87	3	105 \pm 53	3	114 \pm 40	3
8-OH-DPAT	240 \pm 55 [*]	7	239 \pm 35 [*]	7	362 \pm 94 [*]	7
LY53857	346 \pm 97 ⁺	7	193 \pm 30 [*]	7	378 \pm 60 ^{**}	7
DOI	146 \pm 29	4	98 \pm 18	4	162 \pm 61	4
Saline control	100 \pm 19	32	100 \pm 20	32	100 \pm 20	32

^{**} $p < 0.01$;

^{*} $p < 0.05$;

⁺ $p = 0.055$ compared with saline-treated control;

Student's *t*-test.