2001

# Effects of mood stabilizers on transcription factors.

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# THE EFFECTS OF MOOD STABILIZERS ON TRANSCRIPTION FACTORS

by

CAROL ANN GRIMES

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
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ABSTRACT OF DISSERTATION
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One percent of the population suffers from bipolar disorder, a disease that is characterized by the occurrence of severe depression coupled with at least one or more episodes of severe mania. Lithium is the primary therapeutic agent used in the treatment of bipolar disorder, and two anticonvulsants, valproate and carbamazepine, have also emerged as effective treatments of the disease. The therapeutic mechanisms of action of these drugs, as well as the biochemical basis for bipolar disorder, remain unknown. However, the identification of biochemical targets of these drugs may provide clues to the pathogenesis of bipolar disorder and allow the development of more target-specific drugs for the treatment of this disease. The studies presented within this dissertation research examined the effects of lithium, as well as valproate and carbamazepine, on neuronal signaling systems, specifically examining the regulation of transcription factors by two enzymes found to be regulated by lithium: protein kinase C and glycogen synthase kinase-3β. The first study examined the effects of these mood-stabilizing agents on the stimulation of the early growth response-1 transcription factor, a protein reported to be regulated by protein kinase C activation. Findings from this study demonstrated that stimulation of early growth response-1 transcription factor was inhibited by treatment with valproate but was not altered by lithium or carbamazepine. The second

study reviewed the regulation and numerous targets of glycogen synthase kinase-3β and found that the basal levels of four GSK3β-regulated transcription factors--activator protein-1, cyclic AMP response element binding protein, Myc, and β-catenin--were increased, though with different sensitivities, by treatment with lithium. The third study examined the regulation of the cyclic AMP response element binding protein by glycogen synthase kinase-3β and the mood-stabilizing agents lithium and valproate. This study showed, with several experimental paradigms, that the cyclic AMP response element binding protein was negatively regulated by glycogen synthase kinase-3β, an effect that was reversed by treatment with lithium or valproate. Overall, these studies suggest that the inhibtion of glycogen synthase kinase-3β-induced inhibition of transcription factors, including the cyclic AMP response element binding protein, may be important sites for future studies.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Aβ | Amyloid beta protein |
| AP-1 | Activator protein-1 |
| APC | Adenomatous polyposis coli gene product |
| ATP | Adenosine triphosphate |
| CBP | CREB Binding Protein |
| C/EBPα | CCAAT/enhancer binding protein |
| CREB | Cyclic AMP response element binding protein |
| Dvl | Dishevlled |
| EGF | Epidermal growth factor |
| Egr-1 | Early growth response-1 |
| eIF2B | Eukaryotic initiation factor 2B |
| ERK | Extracellular-related kinases |
| Fzl | Frizzled |
| GBP | GSK3β binding protein |
| GSK3β | Glycogen synthase kinase 3β |
| HSF-1 | Heat shock factor-1 |
| IGF-1 | Insulin like growth factor-1 |
| IκB | Inhibitory factor κB |
| Lef | Lymphoid-enhancing factor |
| MAP | Microtubule-associated protein |

| | |
|---|---|
| MEK | MAP kinase kinase |
| NFAT | Nuclear factor of activated T-cells |
| NFκB | Nuclear factor κB |
| PI3K | Phosphatidylinositol 3-kinase |
| Tcf | T cell factor |
| TNFα | Tumor necrosis factor α |
| WT-1 | Wilms' tumor promoter protein |

# INTRODUCTION

One percent of the population of the United States is afflicted with bipolar disorder, which is commonly known as manic-depression (Dixon and Hokin, 1997). This disease is characterized by the manifestation of one or more manic episodes and a history of major depression. Presently, lithium is the primary therapeutic agent used in the treatment of bipolar disorder, and although its therapeutic use is widespread, its mechanism of action remains to be determined (reviewed in Jope, 1999). In addition to lithium, two anticonvulsant drugs, valproate and carbamazepine, have emerged as therapeutic agents in the treatment of bipolar illness (reviewed in Sachs and Thase, 2000). Although both drugs have demonstrated the ability to prevent manic and depressive episodes, their mechanisms of action also remain unknown. The methodologies available to study the biochemical basis of bipolar disorder are limited. Because the underlying cause of bipolar disorder is not known, there are no existing criteria upon which to base the development of animal models of the disease; furthermore, studies in humans are limited to postmortem tissues. Thus, studying the therapeutic mechanism of action of drugs used in the treatment of bipolar disorder is the best available method to gain insight into the pathogenesis of the disease. The fact that three structurally diverse drugs -- lithium, valproate, and carbamazepine -- are therapeutic treatments for this one disease provides a unique advantage, because identifying a specific target common to two or more of the drugs might provide clues to the identity

of therapeutic targets of these agents in bipolar disease. Hopefully, findings from studies of these mood-stabilizing agents will provide more information concerning the underlying cause of bipolar disorder and foster the development of more target-specific drugs with which to treat bipolar disorder.

Numerous investigations over several years support the involvement of lithium in the modulation of cell signaling systems, especially the phosphoinositide signal transduction system (reviewed in Jope and Williams, 1994; Pacheco and Jope, 1996). In the phosphoinositide signaling cascade, phosphatidylinositol is sequentially phosphorylated to ultimately form phosphatidylinositol-4,5-bisphosphate ($PIP_2$), the substrate of phospholipase C. The β-subtype of phospholipase C is a phosphoinositide-specific enzyme under the influence of the G-protein-coupled receptor system. For example, cholinergic muscarinic M1, M3, and M5 receptors are primarily linked to the Gq/11 proteins that mediate activation of phosphoinositide-specific phospholipase C. Activated phospholipase C hydrolyzes $PIP_2$ into two intracellular second messengers: diacylglycerol (DAG), which activates protein kinase C, and inositol-1,4,5-trisphosphate ($IP_3$), which signals the release of intracellularly sequestered calcium. $IP_3$ is subsequently phosphorylated to form inositol-1,3,4,5-tetrakisphosphate or ultimately dephosphorylated to inositol monophosphate, which is dephosphorylated by inositol monophosphatase to free inositol. Lithium has been demonstrated to inhibit inositol monophosphatase with an $IC_{50}$ of 1 mM, a therapeutically relevant concentration (Hallcher and Sherman, 1980). This finding led to the inositol depletion hypothesis (Berridge et al., 1982), which asserted that lithium's inhibition of inositol monophosphatase could result in a reduced supply of inositol available for $PIP_2$ synthesis, resulting in down-regulation of receptor

signaling through the phosphoinositide second messenger pathway. Other studies have extended these findings to include modulation of protein kinase C by lithium (reviewed in Jope and Williams, 1994). As mentioned previously, in the phosphatidylinositol pathway, inositol monophosphate is dephosphorylated by inositol monophosphatase to form free inositol. To complete the phosphoinositide cycle, inositol combines with CDP-diacylglycerol (CDP-DAG) to form phosphatidylinositol. The decreased availability of free inositol caused by lithium's inhibition of inositol monophosphatase results in the accumulation of free CDP-DAG (Stubbs and Agranoff, 1993). This accumulated CDP-DAG is cleaved to form diacylglycerol, which then activates protein kinase C. Thus, these findings indicate that lithium indirectly increases protein kinase C activity by inhibiting inositol monophosphatase. Yuan et al. (1998) proposed that one functional consequence of this activation of protein kinase C by lithium is the stimulation of the DNA binding activity of the AP-1 transcription factor. Numerous reports have found increased AP-1 DNA binding activity in response to lithium in cell culture systems and rat brain (Hedgepeth et al., 1997; Jope and Song, 1997; Ozaki and Chuang, 1997; Asghari et al., 1998; Yuan et al., 1998). Thus, this hypothesis proposes that transcription factors regulated by protein kinase C constitute a group of transcription factors that may be subject to modulation by lithium.

Another example of a protein kinase C-regulated transcription factor is early growth response-1 (Egr-1) (Rupprecht et al., 1994; Ebihara and Saffen, 1997). Egr-1 (also known as NGF1-A, tis8, Krox 24, or zif268) is encoded by the early-growth response gene (*egr-1*) and is a member of a family of immediate early genes that also includes egr-*2*, egr-3, egr-4, and WT-1. Studies investigating Egr-1 expression have

demonstrated stimulation in response to various neuronal signaling systems. Neuronal activation of several glutamate receptor subtypes resulted in increased Egr-1 mRNA expression, and NMDA receptor activity has been implicated in maintaining basal Egr-1 protein expression in the brain (Beckmann and Wilce, 1997). Induction of Egr-1 mRNA has also been reported in multiple cell lines in response to cytokines, growth factors, and some neurotransmitters, and in response to physiological stimulation, stress, and focal brain injury (Beckmann and Wilce, 1997; Ebihara and Saffen, 1997). A recent report using M1 receptor-containing PC12D cells reported stimulation of Egr-1 mRNA levels in response to carbachol, although neither protein levels nor functional activity were examined (Ebihara and Saffen, 1997). Taken together, these studies provide evidence of a role for Egr-1 in neuronal signaling systems, but several important unanswered questions remain that were addressed in this first study of this dissertation research: Do muscarinic receptors coupled to phosphoinositide hydrolysis regulate Egr-1 protein expression and Egr-1 DNA binding activity? Does activation of second messengers, including diacylglycerol and $IP_3$, coupled to muscarinic receptor stimulation, contribute to stimulation of Egr-1 DNA binding activity? Do the mood stabilizers lithium, valproate, or carbamazepine modulate stimulation of Egr-1 DNA binding activity? To address these questions, cholinergic regulation of Egr-1 was examined in human neuroblastoma SH-SY5Y cells that contain endogenous M3 muscarinic receptors coupled to Gq/11 proteins and phosphoinositide hydrolysis.

Protein kinase C-regulated transcription factors are only one category of proteins subject to potential modulation by lithium. A second category of transcription factors that may be regulated by lithium includes those that are regulated by glycogen synthase

kinase-3β (GSK3β). GSK3β is a fascinating enzyme that is involved in multiple signaling systems and is expressed abundantly in the adult brain, indicating a fundamental role for GSK3β in neuronal signaling processes (Leroy and Brion, 1999). GSK3β is regulated by multiple mechanisms including serine and tyrosine phosphorylation, protein complex formation, and intracellular localization. Recent findings also demonstrate that GSK3β is regulated by mood-stabilizing agents. In 1996, it was demonstrated that lithium inhibited GSK3β (Klein and Melton, 1996; Stambolic et al., 1996), with an $IC_{50}$ of 2 mM, a concentration near lithium's therapeutic concentration (0.5 - 1.5 mM) in plasma and brain (Klein and Melton, 1996). Additionally, findings by Chen et al. (1999) showed that valproate, also an effective treatment of bipolar disorder, inhibited GSK3β at concentrations near its therapeutic range. These findings raised the possibility that GSK3β may be a potential target for the therapeutic actions of mood stabilizing drugs, including lithium and valproate.

Although GSK3β was originally identified for its role in glycogen metabolism as an inhibitor of glycogen synthase (Embi et al., 1980), a diverse group of additional substrates of GSK3β have been identified. GSK3β regulates other metabolic proteins including protein phosphatase inhibitor-2 (Hemmings et al., 1982) and pyruvate dehydrogenase (Hoshi et al., 1996), signaling proteins such as nerve growth factor receptor (Taniuchi et al., 1986), and structural proteins including the microtubule-associated protein tau (reviewed in Johnson and Hartigan, 1998). These findings provide evidence of multiple GSK3β substrates, many of which are neuronal proteins, suggesting an important role for GSK3β in the regulation of neuronal signaling. Possibly the largest group of GSK3β substrates are the transcription factors. A large number of transcription

factors are regulated by GSK3β directly through phosphorylation. Findings by Boyle et al. (1991) indicated that GSK3β phosphorylated and inhibited AP-1 DNA binding activity in vitro, and other studies have shown that expression of GSK3β in cultured cells attenuates transcriptional activities of the Jun family of transcription factors (de Groot et al., 1993; Nikolakaki et al., 1993). In 1998, He et al. reported that overexpression of GSK3β resulted in inactivation of HSF-1 following heat shock in HeLa cells, and findings of Bijur and Jope (2000) showed that GSK3β overexpression impaired activation of HSF-1 in response to heat shock in SH-SY5Y human neuroblastoma cells. Two independent studies found that GSK3β phosphorylated and deactivated nuclear factor of activated T-cells (NFAT) in cell culture models (Beals et al., 1997; Graef et al., 1999). GSK3β was also reported to negatively regulate the Myc transcription factor, resulting in decreased cell growth (Henrikkson et al., 1993). Additionally, the regulation of β-catenin has been widely studied, and data demonstrate that GSK3β phosphorylates β-catenin, which prevents its nuclear translocation, thus blocking its activation of transcription of Tcf/Lef target genes (Rubinfeld et al., 1996; Yost et al., 1996). Overall, there is substantial evidence that GSK3β regulates a wide array of transcription factors, providing a substantial number of targets for modulation by lithium. The second report of this dissertation reviewed the regulation of GSK3β, the targets of GSK3β, and examined the potential importance of GSK3β in Alzheimer's disease, apoptotic mechanisms, and the actions of drugs therapeutic for bipolar disorder. In addition, this report compared the activation of four GSK3β-regulated transcription factors -- AP-1, CREB, Myc, and β-catenin -- to modulation by lithium in order to determine whether different transcription factors exhibit different sensitivities to regulation by lithium, and thus GSK3β.

The cyclic AMP response element binding protein (CREB) is a representative member of transcription factors that are regulated by GSK3β and thus potentially subject to modulation by mood-stabilizing drugs such as lithium or valproate. CREB is a basic leucine zipper transcription factor that regulates the expression of genes containing promoters with cyclic AMP response elements (Montminy and Bilezikjian, 1987; Waeber and Habener, 1991; Brindle and Montminy, 1992). CREB is involved in critical cellular processes including formation of long-term memory, maintenance of synaptic plasticity, and apoptosis (Struthers et al., 1991; Davis et al., 1996; Deisseroth et al., 1996). Thus, the regulation of CREB transcription factor activity is critical to numerous cellular functions.

CREB is regulated by complex phosphorylation mechanisms. Multiple kinases activate CREB through phosphorylation at serine-133, triggering the recruitment of the coactivator CREB-binding protein (CBP) and culminating in the transcription of CREB-regulated genes (Gonzalez et al., 1989; Yun et al., 1990; Chrivia et al., 1993). Phosphorylation at serine-133 also creates a consensus site for phosphorylation by GSK3β at serine-129 of CREB (Fiol et al., 1994; Wang et al., 1994). However, only two studies have addressed the consequences of this hierarchical phosphorylation of CREB by GSK3β, and those studies reported opposing findings. In 1994, Fiol et al. reported that GSK3β potentiated cyclic AMP stimulation of CREB in two cell culture models. Bullock and Habener (1998) also reported hierarchical phosphorylation of CREB by GSK3β but demonstrated that GSK3β directed an inhibitory influence on CREB activation. Although it is evident that GSK3β phosphorylates CREB following phosphorylation by a priming kinase, the opposing findings of these two studies leave

questions that remain to be answered, which are addressed in the third study presented herein: Does GSK3β regulate CREB activity? Is this regulation stimulatory or inhibitory toward CREB activation? If GSK3β regulates CREB activity, do the mood stabilizers lithium or valproate reverse the effects of GSK3β on CREB activity?

# CHOLINERGIC STIMULATION OF EARLY GROWTH RESPONSE-1 DNA BINDING ACTIVITY REQUIRES PROTEIN KINASE C AND MITOGEN-ACTIVATED PROTEIN KINASE KINASE ACTIVATION AND IS INHIBITED BY SODIUM VALPROATE IN SH-SY5Y CELLS

by

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Format adapted and errata corrected for dissertation

## ABSTRACT

Activation of muscarinic receptors in human neuroblastoma SH-SY5Y cells with carbachol stimulated a rapid and large increase in early growth response-1 (Egr-1, also called zif268, NGF1-A) protein levels and DNA binding activity. Egr-1 DNA binding activity was stimulated within 15 min of treatment with carbachol and maintained a maximum 20-fold increase over basal between 1 and 2 h after treatment, and the $EC_{50}$ was ~1 μM carbachol. Carbachol-stimulated Egr-1 DNA binding activity was dependent on protein kinase C, as it was potently inhibited by GF109203X ($IC_{50}$ ~0.1 μM) and was reduced by 85 ± 5% by down-regulation of protein kinase C. Inhibitors of increases in intracellular calcium reduced carbachol-induced Egr-1 DNA binding activity by 25-35%. Carbachol-stimulated activation of Egr-1 was reduced 35% by genistein, a tyrosine kinase inhibitor, and 60% by PD098059, an inhibitor of mitogen-activated protein kinases 1/2 (MEK1/2) that activates extracellular-regulated kinases 1/2 (ERK1/2). A novel inhibitory action was caused by chronic (7-day) administration of sodium valproate but not by two other bipolar disorder therapeutic agents, lithium and carbamazepine. Valproate treatment reduced carbachol-stimulated Egr-1 DNA binding activity by 60% but did not alter carbachol-induced activation of ERK1/2 or p38 or increases in Egr-1 protein levels. These results reveal that muscarinic receptors activate Egr-1 through a signaling cascade primarily encompassing protein kinase C, MEK1/2, and ERK1/2 and that valproate substantially inhibits Egr-1 DNA binding activity stimulated by carbachol or protein kinase C.

# INTRODUCTION

Immediate early genes provide a crucial mechanism for rapidly linking signals generated by extracellular molecules to alterations of neuronal function. The early growth response (Egr; also called zif268, krox 24, NGF1-A, and tis8) proteins are a widely distributed family of products of immediate early genes that include Egr-1, Egr-2, Egr-3, and other related proteins (Beckmann and Wilce, 1997). Various stimuli, such as excitatory amino acids, growth factors, and activators of protein kinase C, can induce neuronal expression of Egr proteins, which function as transcription factors with zinc finger DNA binding domains that bind to the common consensus sequence GCG(T/G)GGGCG (Christy and Nathans, 1989; O'Donovan et al., 1999). Several characteristics of Egr-1 suggest that it has a crucial role in neuronal function, as it is regulated by synaptic activity (Worley et al., 1991), has a lower threshold of activation than the prominent immediate early gene product c-fos (Worley et al., 1993), and appears to make an important contribution to neural plasticity, including influencing learning performance (Fordyce et al., 1994).

Cholinergic muscarinic M3 receptors are linked to the phosphoinositide signal transduction system that modulates protein kinase C activity and intracellular calcium levels, and cholinergic function is an integral component of learning and memory (Pacheco and Jope, 1996). These properties suggest that stimulation of muscarinic M3 receptors may influence the activation of Egr, in addition to its well-documented coupling to activation of the AP-1 transcription factor (Jope and Song, 1997). In accordance with this, there have been several reports that activation of muscarinic receptors increases Egr-1 mRNA levels (Arenander et al., 1989; Altin et al., 1991;

Katayama et al., 1993; Hughes and Dragunow, 1994; Coso et al., 1995; Ebihara and Saffen, 1997) and that protein kinase C (Altin et al., 1991; Coso et al., 1995; Ebihara and Saffen, 1997) and influx of extracellular calcium (Ebihara and Saffen, 1997) contribute to this response. Only recently were increases in Egr-1 protein levels and Egr DNA binding activity demonstrated following muscarinic receptor activation in a study of receptor-transfected HEK293 cells, and these responses were partially inhibited by down-regulation of protein kinase C (von der Kammer et al., 1998). Thus, little is known about signaling cascades or modulatory influences regulating this important response to activation of muscarinic receptors.

Modulation of signaling cascades leading to regulation of transcription factor activities and gene expression appears to be important for the therapeutic responses to lithium, carbamazepine, and sodium valproate, agents used in the treatment of bipolar disorder (Jope, 1999). Recent reports have shown that lithium and sodium valproate influence the activation of the immediate early gene transcription factor AP-1 (Williams and Jope, 1995; Chen et al., 1997; Jope and Song, 1997; Ozaki and Chuang, 1997; Unlap and Jope, 1997; Asghari et al., 1998; Yuan et al., 1998), as well as the DNA binding activity of the cyclic AMP response element and NFκB transcription factors (Jope and Song, 1997; Ozaki and Chuang, 1997; Wang et al., 1999). For example, acute treatments of SH-SY5Y cells with lithium and sodium valproate have been reported to increase basal AP-1 DNA binding activity, and lithium inhibited AP-1 activation in response to stimulation of cholinergic muscarinic receptors (Jope and Song, 1997; Asghari et al., 1998; Yuan et al., 1998). Notably lacking are studies of the effects of any of these agents on the activation of Egr or of the effects of chronic administration of these agents, which

is required for therapeutic responses, on the receptor-coupled stimulation of any transcription factor, except for a study of AP-1 in rat brain after pilocarpine administration (Williams and Jope, 1995).

The present investigation was undertaken to address the issues of muscarinic receptor signaling to Egr activation and modulation by agents therapeutic for bipolar disorder. Specifically, we tested if stimulation of endogenous M3 muscarinic receptors in human neuroblastoma SH-SY5Y cells was coupled to activation of Egr DNA binding activity, identified intermediates in the signaling pathway linking muscarinic receptors to activation of Egr-1, and determined if chronic administration of therapeutically relevant concentrations of lithium, carbamazepine, or sodium valproate modulated basal or stimulated Egr DNA binding activity.

## MATERIALS AND METHODS

### *Materials*

Reagents were obtained from the following sources: RPMI 1640 media from Cellgro (Herndon, VA, U.S.A.); horse serum, L-glutamine, and penicillin/streptomycin from Life Technologies (Gaithersburg, MD, U.S.A.); fetal clone II from Hyclone (Logan, UT, U.S.A.); antibodies to Egr-1, Egr-2, and Egr-3 from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); carbachol, phorbol 12-myristate 13-acetate (PMA), diaminobenzidine, genistein, dantrolene, nickel chloride, lithium chloride, carbamazepine, and sodium valproate from Sigma (St. Louis, MO, U.S.A.); GF109203X, Gö6976, and Ro31-8220 from Alexis Biochemicals (San Diego, CA, U.S.A.); BAPTA-AM, SB203580, and PD098059 from CalBiochem (La Jolla, CA, U.S.A.); antibodies to (phospho-ERK1/2) and phospho-p38 from New England Biolabs (Beverly, MA, U.S.A.);

and Lumiglo chemiluminescent substrate from Kirkegaard and Perry Laboratories (Gaithersburg).

*Cell culture*

Human neuroblastoma SH-SY5Y cells were grown on Corning 100-mm-diameter tissue culture dishes (Corning, NY, U.S.A.) in RPMI 1640 medium containing 10% horse serum, 5% fetal clone II, 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in humidified, 37°C chambers with 5% $CO_2$. Cells were plated at a density of $10^5$ cells per 100-mm-diameter dish and were harvested ~48 h later, following treatments described in Results.

*Isolation of nuclear extracts*

SH-SY5Y cells were washed two times with 4 ml of phosphate-buffered saline and lysed with 4 ml Nonidet P-40 lysis buffer [10 m*M* Tris-Cl (pH 7.4), 3 m*M* $MgCl_2$, 10 m*M* NaCl, 0.5% Nonidet P-40]. Cell lysates were centrifuged at 4000 *g* for 5 min at 4°C. The supernatant was discarded, and the pellet containing nuclear material was resuspended in 50 μl of buffer [20 m*M* HEPES (pH 7.9), 20% glycerol, 0.3 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 1 m*M* dithiothreitol, 0.1 m*M* β-glycerophosphate, 0.5 m*M* vanadate, 1 m*M* phenylmethanesulfonyl fluoride, and 1 μg/ml each pepstatin A, leupeptin, and aprotinin]. After a 30-min extraction on ice, samples were centrifuged at 16,000 *g* for 15 min at 4°C. The supernatant containing nuclear extracts was transferred to a sterile microfuge tube, and protein concentrations were determined by the method of Bradford (1976).

*Electrophoretic mobility shift assay (EMSA)*

A 22-bp double-stranded oligonucleotide containing the consensus sequence for Egr 5'-(ATGCCCGGCGCGGGGGCGAGGG)-3' was used for EMSAs (Gibco, Grand Island, NY, U.S.A.). Double-stranded oligonucleotide (200 pmol) was radiolabeled by incubating for 1 h at 37°C in 20 µl containing 10x Gibco React 2 buffer, 0.5 m*M* dNTP, DNA polymerase I (Klenow enzyme; Gibco), and 100 µCi of [$\alpha^{32}$P]-dCTP (Amersham, Arlington Heights, IL, U.S.A.). Following incubation, samples were diluted to 100 µl with sterile TE buffer [10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA], and free probe was removed by centrifugation at 1000 rpm for 45 s on a Sephadex G-50 column.

DNA binding was measured by incubating nuclear extracts (10 µg of protein) in 20 µl of binding buffer containing 20 m*M* HEPES (pH 7.0), 4% glycerol, 500 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 1 mg of poly(dIdC), and ~10,000 cpm of radiolabeled Egr oligonucleotide for 30 min at 4°C. For supershift experiments, nuclear extracts were incubated with antibody (0.5 µg) to Egr-1, Egr-2, or Egr-3 for 30 min before incubation with binding buffer. Reaction mixtures were electrophoresed on 6% nondenaturing polyacrylamide gels in 0.25X TBE (22.3 mM Tris, 22.3 m*M* boric acid, and 0.5 mM EDTA) for 1.5 h at 150 V. The gels were then vacuum-dried, exposed to a phosphorscreen overnight, and quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

*Immunoblotting*

Nuclear extracts (25 µg) were mixed with Laemmli sample buffer (2% sodium dodecyl sulfate) and placed in a boiling water bath for 10 min. Proteins were resolved in

7.5% sodium dodecyl sulfate polyacrylamide gels, transferred to nitrocellulose, and incubated with polyclonal Egr-1, Egr-2, or Egr-3 antibody (1:1,000). Immunoblots were developed using horseradish peroxidase-conjugated secondary antibody followed by incubation in diaminobenzidine solution. Phosphotyrosine immunoblots were incubated with polyclonal phospho-ERK1/2 or phospho-p38 antibody and were developed using horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence.

*Statistical analysis*

All treatments were tested at least three times with separate batches of cells, and statistical significance was determined by ANOVA using the INSTAT program.

## RESULTS

*Stimulation of muscarinic receptors increases Egr-1 DNA binding activity*

To investigate the effects of cholinergic stimulation on Egr activation, DNA binding activity was measured in nuclear extracts prepared from human neuroblastoma SH-SY5Y cells that had been treated with the cholinergic agonist carbachol (1 m*M* for 1 h). Carbachol treatment significantly increased Egr DNA binding activity (Fig. 1). An antibody specific for Egr-1 completely supershifted the carbachol-stimulated band to one of slower mobility, whereas antibodies specific for Egr-2 and Egr-3 only slightly diminished the band intensity. Therefore, carbachol stimulated Egr DNA binding activity in SH-SY5Y cells, and Egr-1 was the primary protein in the complex.

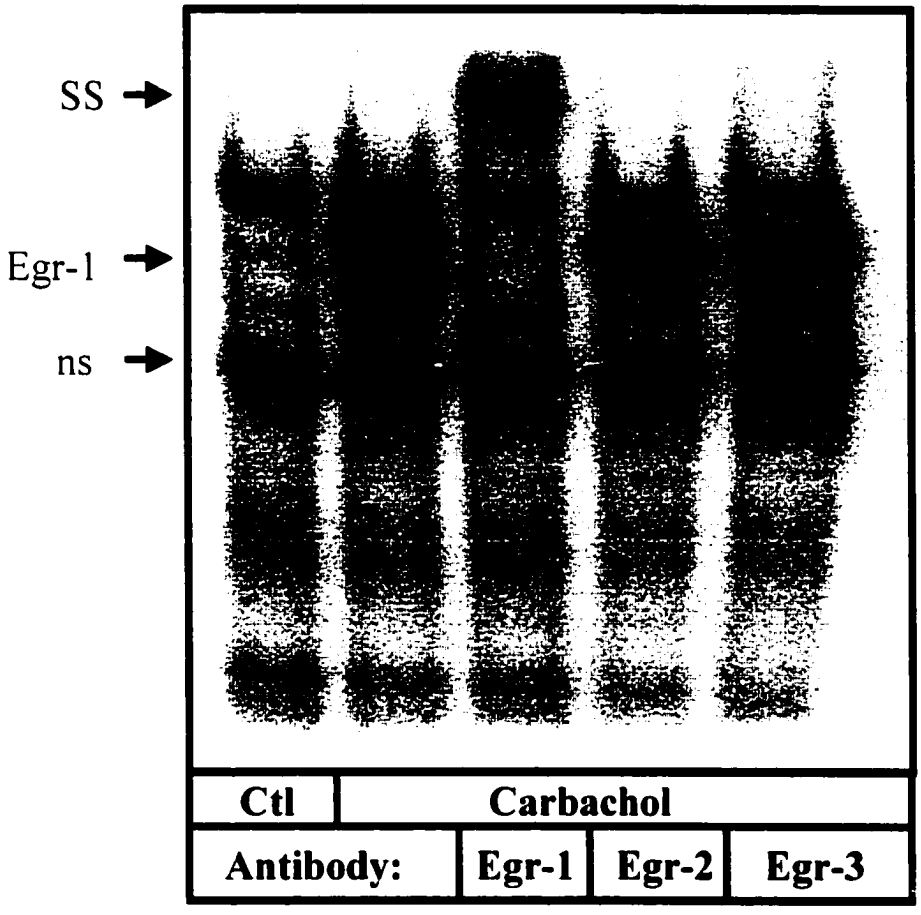



**FIG. 1.** EMSA and supershift analysis of Egr DNA binding activity in SH-SY5Y cells. SH-SY5Y cells were treated without (Ctl) or with 1 m*M* carbachol for 1 h, nuclear extracts from carbachol-treated cells were preincubated with antibodies for Egr-1, Egr-2, or Egr-3, and Egr DNA binding activity was measured using EMSA analysis as described in Materials and Methods. Egr-1 DNA binding activity, the supershifted (SS) band, and a nonspecific (ns) band are indicated with arrows.

Figure 2 shows the time course (15 min-6 h) of carbachol-stimulated Egr-1 DNA binding activity. Stimulation of Egr-1 DNA binding activity was evident within 15 min of treatment with carbachol, a maximal increase that was ~2,000% of control values was maintained between 1 and 2 h of treatment, and this was followed by a rapid decline to ~500% of control following 3 h of treatment with carbachol. Preincubation with the muscarinic antagonist atropine (10 $\mu M$) completely blocked carbachol-stimulated Egr-1 DNA binding activity, confirming that Egr-1 activated in response to carbachol was due to stimulation of muscarinic receptors.

The relationship between Egr-1 DNA binding and Egr-1 protein levels was examined by using quantitative immunoblots to determine the effects of carbachol on Egr-1 protein levels. Carbachol treatment increased Egr-1 protein levels in whole-cell lysates (data not shown) and in nuclear extracts (Fig. 3A and B), and the time course of this increase paralleled that of Egr-1 DNA binding after carbachol treatment. Although a labeled band corresponding to Egr-3 DNA binding activity was not observed in the EMSAs, carbachol stimulated a large increase in the level of Egr-3 in nuclear extracts (Fig. 3C). Egr-2 was not detected in immunoblots of nuclear extracts with or without carbachol treatment (data not shown).

The concentration dependence of carbachol-stimulated Egr-1 DNA binding activity was investigated in SH-SY5Y cells. Preliminary experiments indicated that maximal Egr-1 DNA binding activity was induced by carbachol concentrations between 10 and 100 $\mu M$; therefore, carbachol concentrations of 0.1, 1, 3, 10, and 100 $\mu M$ were used. These experiments revealed that the $EC_{50}$ for carbachol-stimulated Egr-1 DNA

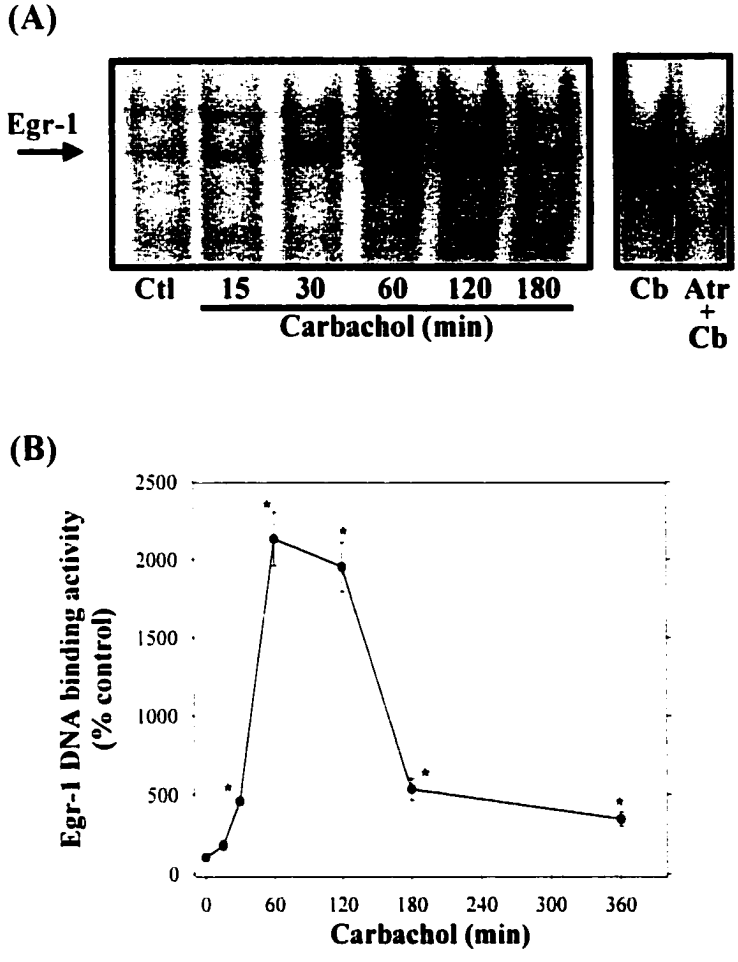



**FIG. 2.** Time course of carbachol (Cb)-stimulated Egr-1 DNA binding activity. Egr-1 DNA binding activity was measured by EMSA analysis in nuclear extracts from SH-SY5Y cells incubated with 1 m*M* carbachol for 15, 30, 60, 120, 180, and 360 min. **A:** A representative EMSA of the time course of Cb-stimulated Egr-1 DNA binding activity. Ctl, untreated control. Also shown is a representative EMSA demonstrating that 10 μ*M* atropine (Atr) blocked Cb-stimulated Egr-1 DNA binding activity (measured 1 h after treatment). **B:** Quantitation of Egr-1 DNA binding activity. Values are given as percentages of Egr-1 DNA binding activity in Ctl cells. Data are mean ± SEM (bars) values (n = 3). $*p < 0.05$ compared with Ctl cells.

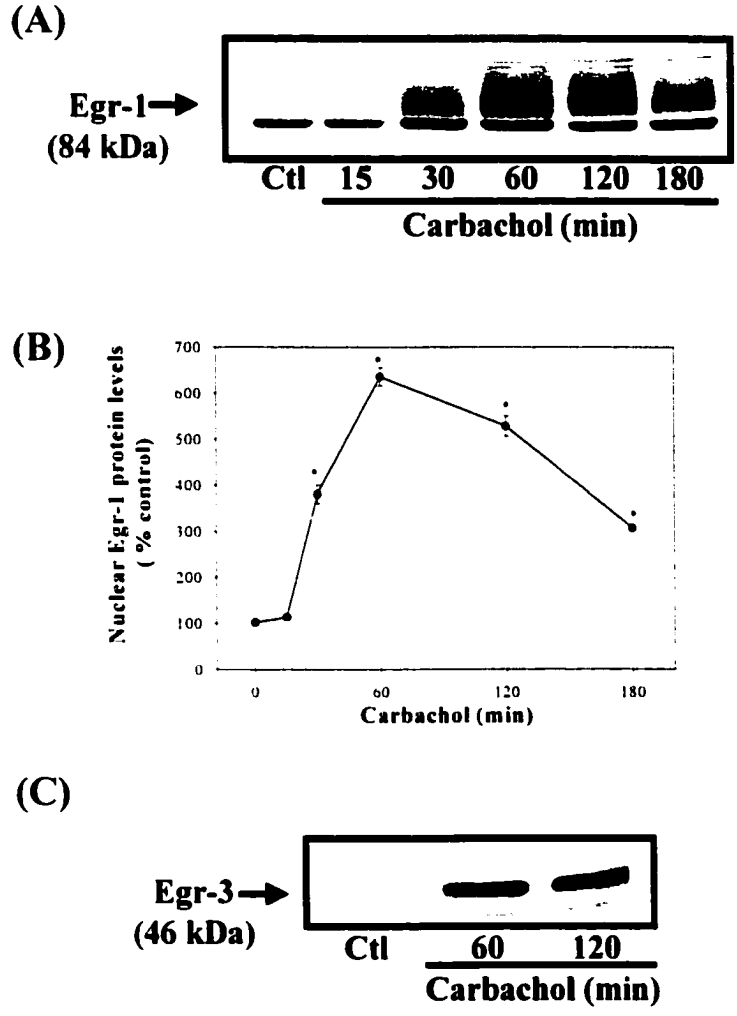



**FIG. 3.** Egr-1 protein levels increase after carbachol treatment. Nuclear extracts were prepared from SH-SY5Y cells treated with 1 m*M* carbachol for 0, 15, 30, 60, 120, or 180 min. **A:** A representative immunoblot of carbachol-induced nuclear Egr-1 protein levels compared with untreated control (Ctl) cells. **B:** Quantitation of carbachol-stimulated Egr-1 protein levels in nuclear extracts. Data are mean ± SEM (bars) values (n = 4). $*p < 0.05$ compared with Ctl cells. **C:** An immunoblot of carbachol-induced nuclear Egr-3 protein levels.

binding activity was ~1 μ*M*, and maximal stimulation was achieved following treatment with ~10 μ*M* carbachol (Fig. 4).

*Signaling intermediates mediating carbachol-stimulated Egr-1 DNA binding activity*

The roles of the second messengers produced by the muscarinic receptor-coupled phosphoinositide signaling system were examined in the mediation of carbachol-stimulated Egr-1 DNA binding activity and protein levels in SH-SY5Y cells. The involvement of protein kinase C, which is activated by stimulation of the phosphoinositide signaling system, was investigated initially using the protein kinase C activator PMA and the inhibitor GF109203X. Exposure of cells to 0.5 μ*M* PMA for 1 h resulted in a substantial increase in Egr-1 DNA binding activity, and this was completely blocked by pretreatment (5 min) with 10 μ*M* GF109203X (Fig. 5A and B). GF109203X also concentration-dependently decreased carbachol-induced Egr-1 DNA binding activity, with the low concentration of 0.1 μ*M* GF109203X causing a 45% inhibition of the response to carbachol and 10 μ*M* GF109203X causing complete blockade. Immunoblots revealed that GF109203X also inhibited carbachol- and PMA-stimulated increases in nuclear Egr-1 protein levels (Fig. 5C). Carbachol-induced increases in Egr-1 DNA binding activity also were reduced by other treatments that inhibit protein kinase C. These include the selective inhibitors Ro31-8220 and Gö6976, as well as down-regulation of protein kinase C caused by a 24-h pretreatment with 1 μ*M* PMA (Fig. 5D). The effects of PMA-induced down-regulation of protein kinase C are especially notable because this treatment may be the most reliable for specifically reducing protein kinase C activity, and it inhibited carbachol-stimulated Egr-1 DNA binding activity by 85 ± 5% (n

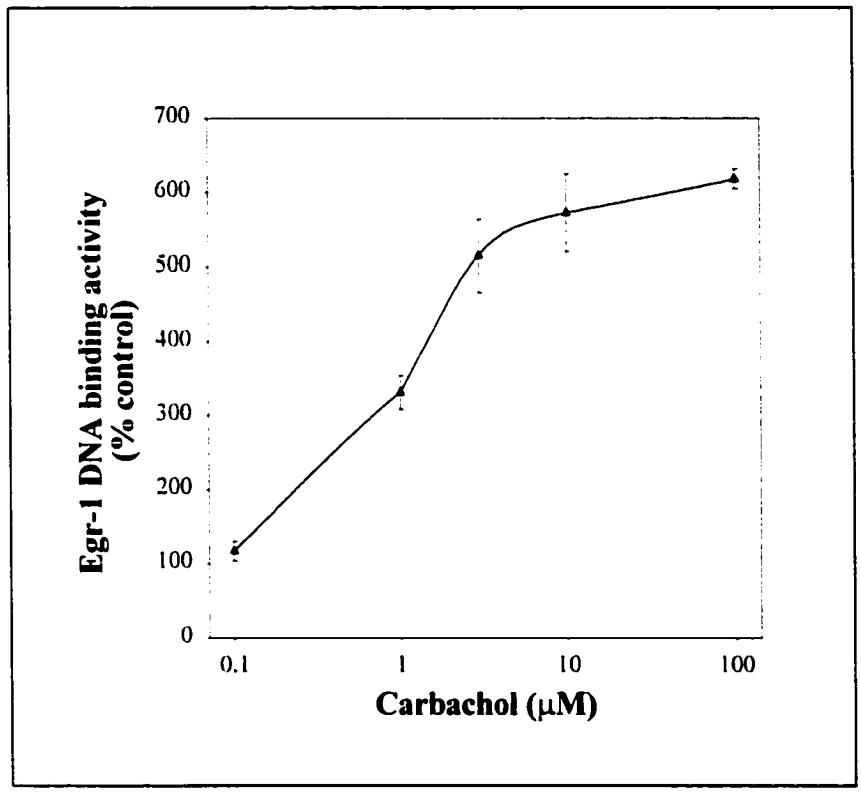
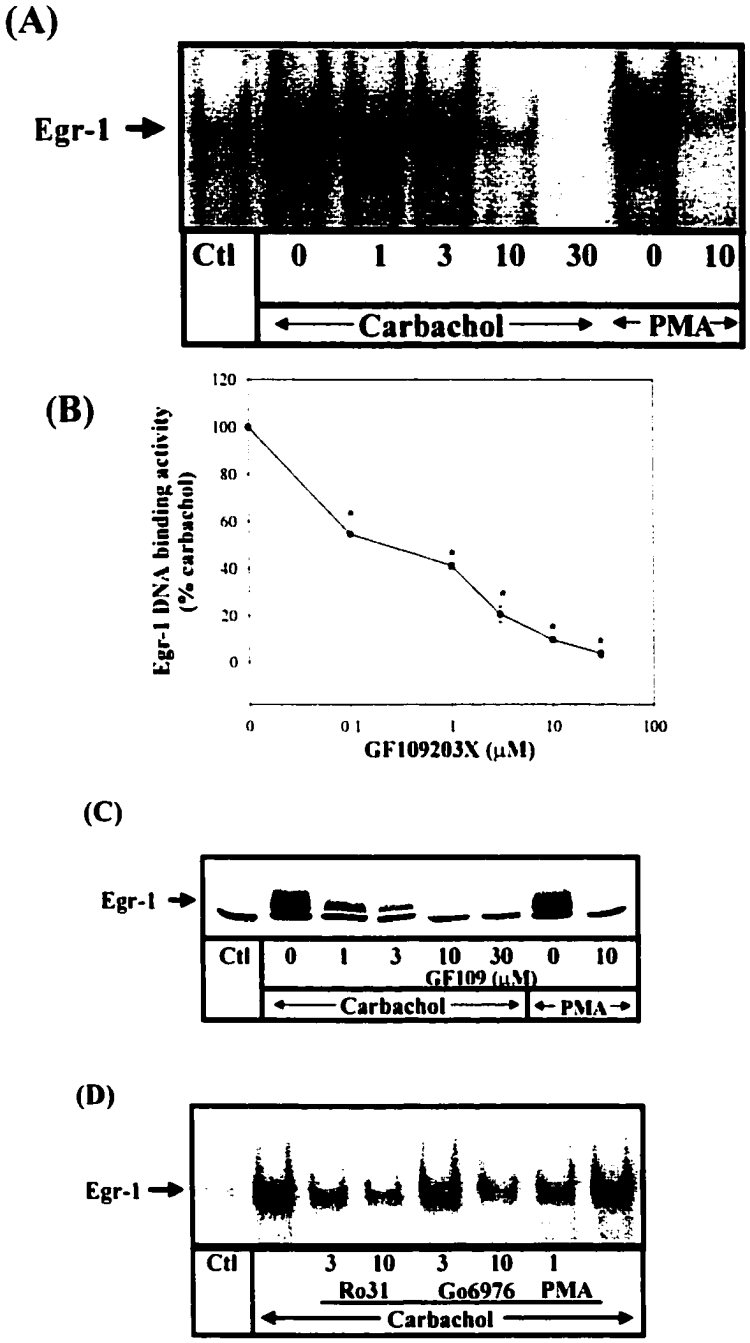



**FIG. 4.** Carbachol concentration-dependent stimulation of Egr-1 DNA binding activity. Egr-1 DNA binding activity was measured by EMSA analysis in nuclear extracts from SH-SY5Y cells incubated with 0.1, 1, 3, 10, or 100 μ*M* carbachol for 1 h. Values are given as percentages of Egr-1 DNA binding activity in untreated cells (controls). Data are mean ± SEM (bars) values ($n = 5$).

**FIG. 5.** Inhibition of protein kinase C blocks carbachol- and PMA-induced Egr-1 DNA binding activity and increases nuclear Egr-1 protein levels. **A-C:** Egr-1 DNA binding activity and nuclear Egr-1 levels were examined by EMSA and immunoblot analysis, respectively, in nuclear extracts prepared from SH-SY5Y cells pretreated with 0, 1, 3, 10, or 30 $\mu M$ GF109203X for 5 min followed by incubation with 1 m$M$ carbachol or 0.5 $\mu M$ PMA for 1 h. **A:** A representative EMSA of the inhibition by GF109203X of carbachol- and PMA-induced Egr-1 DNA binding activity. Ctl, untreated control. **B:** Quantitation of the effects of GF109203X on carbachol-stimulated Egr-1 DNA binding activity. Values are given as percentages of Egr-1 DNA binding activity in cells treated with carbachol alone. Data are mean ± SEM (bars) values (n = 3-5). $*p < 0.05$ compared with carbachol-stimulated Egr-1 DNA binding activity. **C:** A representative immunoblot demonstrating the inhibition by GF109203X of carbachol- and PMA-induced nuclear Egr-1 protein levels. **D:** Egr-1 DNA binding activity was measured in cells treated with the protein kinase C inhibitors Ro31-8220 (Ro31; 15 min) or Gö6976 (15 min) at 3 and 10 $\mu M$ or after protein kinase C down-regulation induced by a 24-h treatment with 1 $\mu M$ PMA, followed by incubation with 1 m$M$ carbachol (1 h).



= 5). These findings demonstrate that protein kinase C activity is necessary for carbachol-stimulated Egr-1 DNA binding activity.

The role of calcium was examined in the Egr-1 response to carbachol treatment because intracellular calcium concentrations are increased following stimulation of the phosphoinositide signaling system. As shown in Fig. 6, preincubation of SH-SY5Y cells with BAPTA-AM (20 μ*M*) caused a 35% reduction in carbachol-induced Egr-1 DNA binding activity. Also, pretreatment with dantrolene (1 μ*M*), an inhibitor of calcium release from the endoplasmic reticulum, had no effect on the Egr-1 response. Pretreatment with nickel chloride (2 m*M*), an inhibitor of agonist-induced calcium influx, or KN-62, an inhibitor of calcium/calmodulin-dependent kinase II, caused 25-32% reductions in carbachol-induced Egr-1 DNA binding activity. These results indicate that an increased intracellular calcium is necessary for optimal activation of Egr-1 by carbachol, although this response demonstrated a greater dependence on protein kinase C activity.

The involvement of protein tyrosine kinase activity and mitogen-activated protein (MAP) kinases in the Egr-1 response to carbachol was examined using selective protein kinase inhibitors. The tyrosine kinase inhibitor genistein (100 μ*M* for 10 min) significantly attenuated carbachol-stimulated Egr-1 DNA binding activity (Fig. 7A). PD098059 is an inhibitor of MAP kinase kinase1/2 (MEK1/2), which activates extracellular-related-kinases 1 and 2 (ERK1/2). Examination of the inhibition of ERK1/2 activation by 10, 25, and 50 μ*M* PD098059 revealed that 50 μ*M* PD098059 was necessary to obtain substantial inhibition (Fig. 7B) and 50 μ*M* PD098059 caused a 60% inhibition of carbachol-stimulated Egr-1 DNA binding activity. However, pretreatment

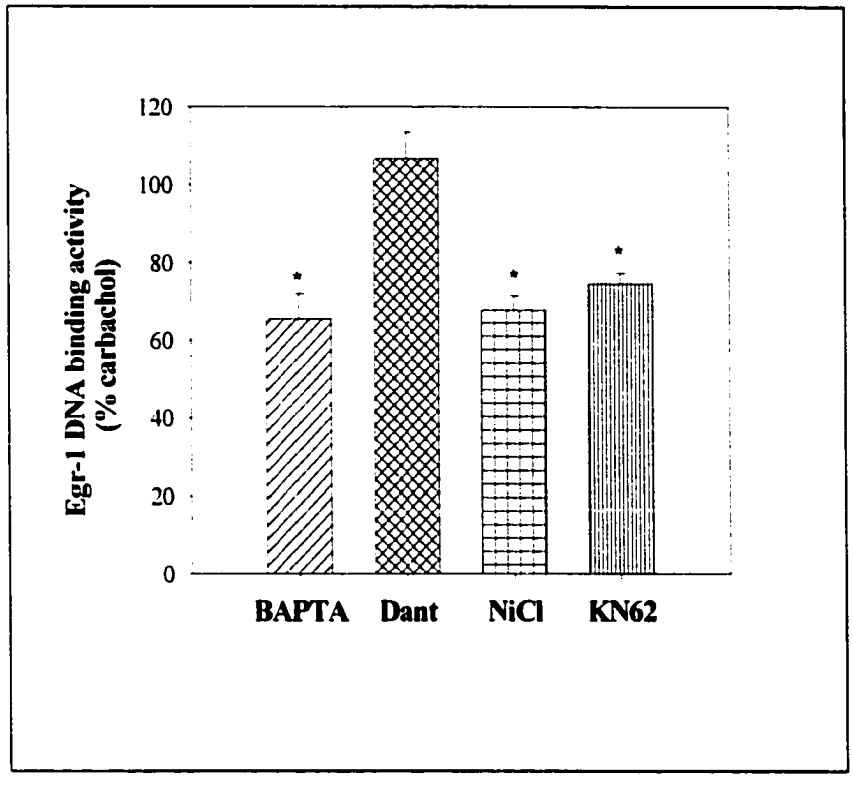



**FIG. 6.** Effects of calcium modulators on carbachol-stimulated Egr-1 DNA binding activity. Egr-1 DNA binding activity was measured in nuclear extracts from SH-SY5Y cells preincubated with 20 μ*M* BAPTA-AM (BAPTA; for 30 min), 1 μ*M* dantrolene (Dant; for 10 min), 2 m*M* nickel (NiCl; for 10 min), or 30 μ*M* KN-62 (for 10 min), followed by incubation with carbachol (1 m*M* for 1 h). Values are given as percentages of Egr-1 DNA binding activity in carbachol-treated cells. Data are mean ± SEM (bars) values (n = 3). **p* < 0.05 compared with carbachol-stimulated Egr-1 DNA binding activity.

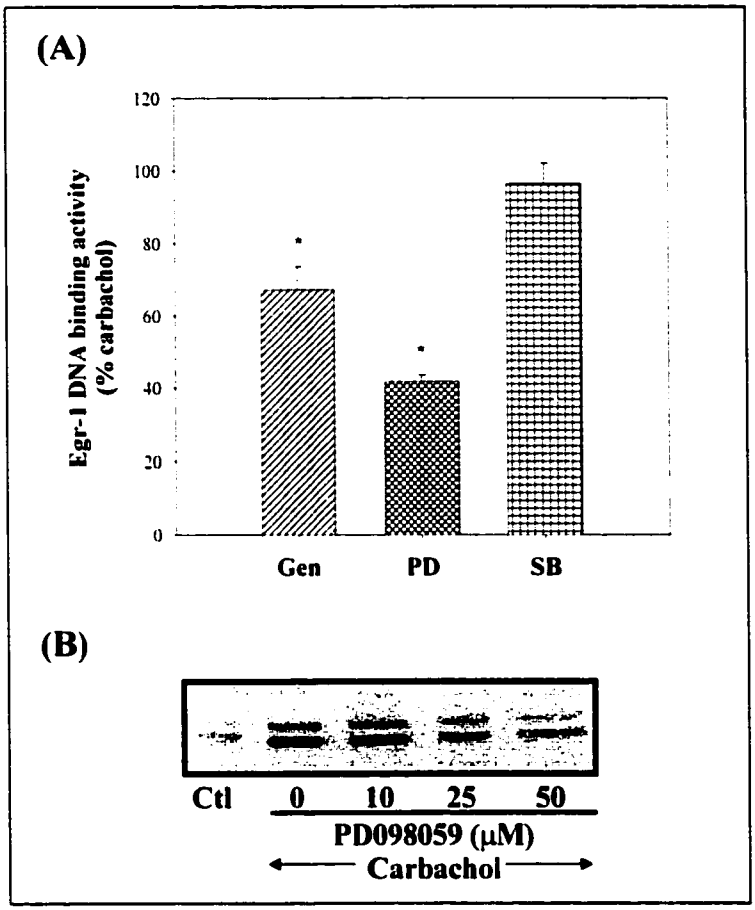



**FIG. 7.** Signaling intermediates mediating carbachol-stimulated Egr-1 DNA binding activity. **A:** Egr-1 DNA binding activity was measured by EMSA analysis in nuclear extracts from SH-SY5Y cells pretreated for 10 min with 100 μ*M* genistein (Gen), 50 μ*M* PD098059 (PD), or 20 μ*M* SB203580 (SB) followed by incubation with 1 m*M* carbachol for 1 h. Values are given as percentages of Egr-1 DNA binding activity in carbachol-treated cells. Data are mean ± SEM (bars) values (n = 3-5) $*p < 0.05$ compared with carbachol-stimulated Egr-1 DNA binding activity. **B:** A representative immunoblot (n = 3-4) of ERK1/2 phosphotyrosine immunoreactivity measured in cell lysates from cells pretreated with 0, 10, 25, or 50 μ*M* PD (for 30 min) followed by incubation with 1 m*M* carbachol for 15 min. Ctl, untreated control.

with SB203580 (20 μ*M* for 10 min), an inhibitor of p38, had no effect on carbachol-stimulated Egr-1 DNA binding activity. These findings indicate that ERK1/2 activation is necessary for full muscarinic-receptor stimulated activation of Egr-1.

*Chronic sodium valproate treatment inhibits stimulated Egr-1 DNA binding activity*

The effects of chronic (7-day) pretreatment with therapeutically relevant concentrations of lithium, carbamazepine, or sodium valproate on carbachol-stimulated Egr-1 DNA binding activity were examined. Chronic treatment with lithium (1 m*M*) or carbamazepine (0.05 m*M*) did not alter carbachol-stimulated Egr-1 DNA binding activity (Fig. 8). However, chronic treatment with sodium valproate (0.5 m*M*) caused a large inhibition (60 ± 5%) of carbachol-stimulated Egr-1 DNA binding activity. This was not due to a direct effect of sodium valproate on DNA binding by Egr-1 because inclusion of 0.5 m*M* valproate in the binding assay with nuclear extracts from carbachol-treated cells did not alter Egr-1 DNA binding (data not shown). As expected from the EMSA results, neither pretreatment with neither lithium nor carbamazepine altered carbachol-stimulated increases in Egr-1 protein levels in nuclear extracts (data not shown). Unexpectedly, however, sodium valproate treatment also did not affect increases in the nuclear Egr-1 protein levels induced by carbachol in the same samples in which valproate drastically reduced Egr-1 DNA binding activity (Fig. 9A).

Because protein kinase C was found to play a predominant role in mediating carbachol-stimulated activation of Egr-1 DNA binding activity, the effects of sodium valproate were tested on protein kinase C-activated Egr-1 to determine if its inhibitory

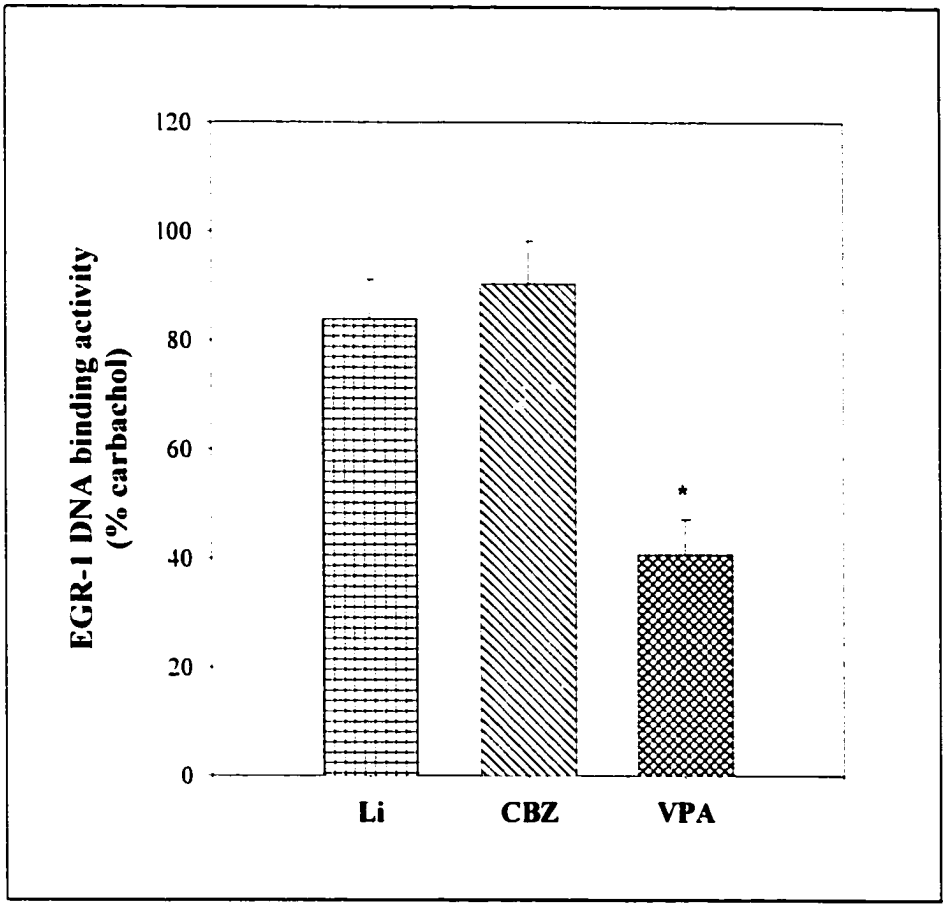



**FIG. 8.** Effects of chronic treatment with lithium (Li), carbamazepine (CBZ), or sodium valproate (VPA) on carbachol-stimulated Egr-1 DNA binding activity. Egr-1 DNA binding activity was measured in nuclear extracts from SH-SY5Y cells treated with 100 $\mu M$ carbachol with or without 7-day pretreatments with Li, CBZ, or VPA. Values are given as percentages of Egr-1 DNA binding activity stimulated by carbachol in cells not treated with antibipolar drugs. Data are mean ± SEM (bars) values (n = 5). $*p < 0.05$ compared with carbachol-stimulated Egr-1 DNA binding activity.

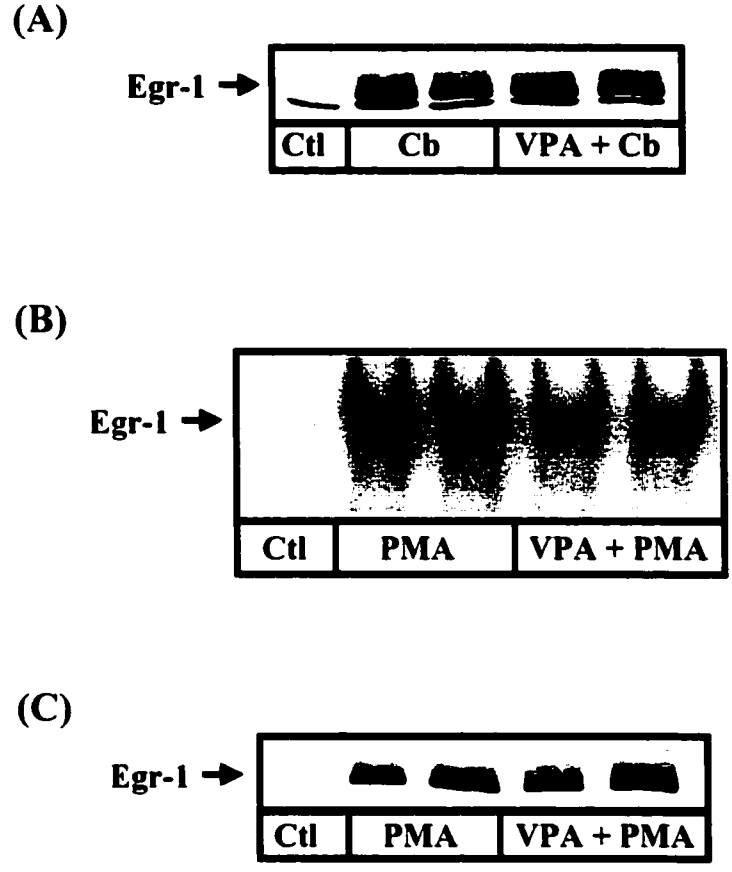



**FIG. 9.** Effects of chronic sodium valproate (VPA) treatment on carbachol (Cb)-induced nuclear Egr-1 protein levels and PMA-induced Egr-1 DNA binding activity and nuclear protein levels. Egr-1 DNA binding activity and nuclear Egr-1 levels were measured by EMSA and immunoblot analysis, respectively, in SH-SY5Y cells treated chronically with VPA. **A:** A representative immunoblot (n = 3) of nuclear Egr-1 levels from untreated control (Ctl) cells or cells pretreated with 0.5 m*M* VPA for 7 days followed by incubation with 1 m*M* Cb for 1 h. **B** and **C:** A representative EMSA (n = 4) and immunoblot (n = 3), respectively, of Ctl cells compared with cells pretreated with 0.5 m*M* VPA for 7 days followed by incubation with 0.5 μ*M* PMA for 1 h.

effect on carbachol-stimulated Egr-1 DNA binding activity was targeted upstream or downstream of protein kinase C. Chronic valproate treatment (0.5 m*M* for 7 days) reduced PMA-induced Egr-1 DNA binding activity by 50 ± 6% (Fig. 9B). However, similar to results obtained for carbachol-stimulated Egr-1, valproate did not alter PMA-induced nuclear Egr-1 protein levels (Fig. 9C).

To determine if valproate inhibited carbachol-stimulated Egr-1 DNA binding activity upstream or downstream of MAP kinases, the phosphotyrosine immunoreactivities of ERK1/2 and p38 were examined. Treatment with carbachol stimulated large increases in the phosphotyrosine immunoreactivities of ERK1/2, a response that was unaltered by chronic valproate treatment, and p38, which was significantly increased in cells treated chronically with valproate (Fig. 10). Thus, the inhibition by sodium valproate of carbachol-stimulated Egr-1 DNA binding activity cannot be attributed to an inhibitory effect of valproate on the activation of ERK1/2 or p38.

## DISCUSSION

Transcription factors are pivotal in cell signaling processes, relaying signals generated by extracellular agents to the nucleus to regulate gene expression. Egr-1 is one of a limited number of transcription factors encoded by immediate early genes that can be activated very rapidly (Beckmann and Wilce, 1997; O'Donovan et al., 1999), and it has been shown to be linked to synaptic activity and neural plasticity (Worley et al., 1991, Fordyce et al., 1994). The present study demonstrated that muscarinic receptor stimulation induces a rapid and robust increase in Egr-1 protein levels and Egr-1 DNA

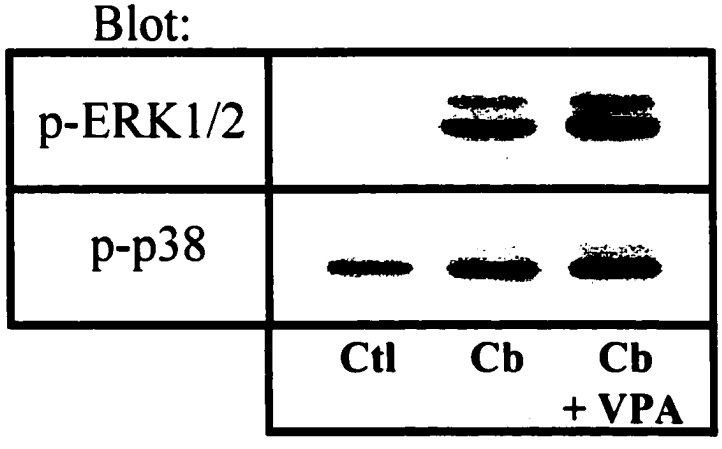



**FIG. 10.** Effect of chronic valproate (VPA) treatment on ERK1/2 and p38 phosphotyrosine immunoreactivities. ERK1/2 and p38 phosphotyrosine immunoreactivities were measured by immunoblot analysis of cell lysates prepared from SH-SY5Y cells pretreated with 0.5 m*M* VPA for 7 days followed by incubation with 1 m*M* carbachol (Cb) for 15 min. Chronic VPA treatment did not significantly alter activation of ERK1/2, but the carbachol-stimulated increase in p38 phosphotyrosine immunoreactivity was 112% greater ($p < 0.05$; n = 5) after treatment with chronic sodium VPA. Ctl, untreated control.

binding activity and that this stimulation is dependent on the activation of protein kinase C, increased intracellular calcium, tyrosine kinase activity, and the MAP kinase signaling cascade. Furthermore, a therapeutically relevant pretreatment with sodium valproate was found to drastically inhibit stimulated Egr-1 DNA binding activity.

Muscarinic receptor stimulation has been reported to induce Egr-1 mRNA levels in rat astrocytes (Arenander et al., 1989), PC12 cells (Altin et al., 1991), NG108-15 cells (Katayama et al., 1993), receptor-transfected NIH 3T3 cells (Coso et al., 1995), PC12D cells (Ebihara and Saffen, 1997), and receptor-transfected HEK293 cells (von der Kammer et al., 1998). The present study demonstrated that the time course of muscarinic receptor-stimulated increases in Egr-1 protein levels and DNA binding activity was more prolonged than that of previously reported increases in Egr-1 mRNA levels. For example, in astrocytes, PC12D cells, and NIH 3T3 cells, muscarinic receptor stimulation increased the Egr-1 mRNA level maximally after 1 h, but within 2 h it had returned to the basal level (Arenander et al., 1989; Coso et al., 1995; Ebihara and Saffen, 1997). In contrast, it is evident that a much longer functional response was attained by muscarinic receptor stimulation, as Egr-1 DNA binding activity remained significantly elevated for at least 6 h in carbachol-stimulated SH-SY5Y cells. Thus, muscarinic receptor stimulation causes a rapid and long-lasting activation of Egr-1.

The signaling intermediates involved in muscarinic receptor stimulation of Egr-1 have not been definitively established. Muscarinic receptor stimulation of Egr-1 mRNA levels was reported to require protein kinase C activation (Altin et al., 1991, Coso et al., 1995, Ebihara and Saffen, 1997) and the influx of extracellular calcium (Ebihara and Saffen, 1997). von der Kammer et al. (1998) found that carbachol-stimulated Egr-1

mRNA and protein levels both are at least partially dependent on protein kinase C activation. In agreement with this, the protein kinase C inhibitor GF109203X potently inhibited carbachol-induced increases in Egr-1 protein levels, and this paralleled the inhibition of carbachol-stimulated Egr-1 DNA binding activity. Other inhibitors of protein kinase C also blocked activation of Egr-1 stimulated by carbachol, with PMA-induced down-regulation of protein kinase C causing almost complete inhibition of this response. Increases in intracellular calcium also were necessary for optimal activation of Egr-1, but this response was less dependent on calcium than on protein kinase C, as maximal reductions of 25-35% were obtained with calcium inhibitors. Although inhibition of calcium/calmodulin-dependent kinase II by KN-62 was reported to block calcium-induced Egr-1 mRNA expression in PC12 cells (Enslen and Soderling, 1994) and in ELM-I-1 cells (Schaefer et al., 1998), KN-62 reduced carbachol-stimulated Egr-1 DNA binding activity by 25%. These results indicate that muscarinic receptor stimulation of Egr-1 DNA binding activity and nuclear protein levels is highly dependent on protein kinase C activation, but less dependent on calcium mobilization, in human neuroblastoma SH-SY5Y cells.

Muscarinic receptor stimulation activates multiple intermediary kinases, such as MEK, ERK1/2, and p38 (Gutkind, 1998). The tyrosine kinase inhibitor genistein reduced carbachol-stimulated Egr-1 DNA binding activity, demonstrating the necessity for tyrosine kinase activation in muscarinic receptor-linked activation of Egr-1. This is in accordance with a study by Humblot et al. (1997) demonstrating that increased Egr-1 mRNA levels and DNA binding activity in response to stimulation of $5HT_2$ receptors, which are coupled to the phosphoinositide signal transduction system, require protein

tyrosine kinase activity in PC12 cells. PD098059, an inhibitor of MEK1/2, recently was reported to attenuate growth hormone-induced Egr-1 gene expression in 3T3-F442A cells (Hodge et al., 1998) and inhibited lysophosphatidic acid-induced Egr-1 mRNA expression in rat mesangial cells (Reiser et al., 1998). In SH-SY5Y cells, PD098059 significantly reduced carbachol-stimulated Egr-1 DNA binding activity and nuclear protein levels. SB203580, an inhibitor of p38, reduced anisomycin-induced Egr-1 mRNA expression in NIH3T3 cells (Lim et al., 1998) but had no effect on lysophosphatidic acid-induced Egr-1 mRNA levels in PC12 cells (Hodge et al., 1998) or on carbachol-stimulated Egr-1 DNA binding activity or nuclear protein levels in this study. Taken together, these results indicate that activation of MEK1/2, which is coupled to activation of ERK1/2, but not of p38 kinase, is required for stimulation of Egr-1 DNA binding activity in response to carbachol in human neuroblastoma SH-SY5Y cells.

Evidence obtained during the last few years indicates that modulation of signaling cascades, transcription factor activities, and gene expression may contribute to the therapeutic effects of drugs used to treat bipolar disorder, which include lithium, sodium valproate, and carbamazepine (Jope, 1999). Although inhibitory actions of these drugs on transcription factors (predominantly AP-1) have been reported, as described in the introductory section, no reports have appeared concerning activation of Egr-1 or direct receptor-coupled activation of any transcription factor after chronic administration of any of these agents, although chronic administration is necessary for therapeutic responses. This study found that chronic treatment with a therapeutically relevant concentration of sodium valproate greatly reduced carbachol-induced Egr-1 DNA binding activity, which is in marked contrast to the lack of effects of lithium or carbamazepine. Furthermore, a

novel inhibitory mechanism accounted for this effect of valproate, as it did not inhibit the signaling cascade originating at the muscarinic receptor and encompassing activation of protein kinase C and ERK1/2, which led to increased levels of Egr-1 protein, but the functional DNA binding activity of Egr-1 following stimulation of muscarinic receptors or of protein kinase C was markedly inhibited. Although the mechanisms that regulate the competency of Egr-1 binding to DNA remain to be identified, this effect of valproate provides a precise mechanism whereby Egr-1 activity can be regulated by valproate in isolation from other downstream effectors of the muscarinic receptor-coupled signaling cascades.

Although activation of muscarinic receptors linked to the phosphoinositide signal transduction system stimulates both AP-1 and Egr-1 DNA binding activities in SH-SY5Y cells, some intriguing differences in the signaling cascades and regulation of these responses have been revealed. Identical treatments with nickel, which blocks the plateau phase of carbachol-induced increases in intracellular calcium, or KN-62, an inhibitor of calcium/calmodulin kinase II, cause at least twice as great an inhibition of carbachol-induced AP-1 than of Egr-1 DNA binding activity (Jope and Song 1997; Pacheco and Jope, 1998). Thus activation of Egr-1 appears less dependent than that of AP-1 on the calcium arm of the phosphoinositide signaling system in SH-SY5Y cells. These findings are in accordance with a previous report that muscarinic receptor-coupled activation of c-Jun kinase displayed a much greater dependence on modulation by increased intracellular calcium than did activation of ERK1/2 (Mitchell et al., 1995). Also, whereas 7 days of treatment with sodium valproate greatly diminished carbachol-induced Egr-1 DNA binding activity, but lithium and carbamazepine were ineffective, carbachol-induced AP-

1 DNA binding activity was unaffected by 7 days of treatment with sodium valproate but was inhibited by ≥ 50% by 7 days of treatment with lithium or carbamazepine (Pacheco and Jope, 1998). Thus, the three bipolar disorder therapeutic agents each inhibit muscarinic receptor-mediated transcription factor activation but with varying selectivities for Egr-1 and AP-1.

In conclusion, this study presents two novel findings concerning Egr-1 activation. First, the results define a signaling pathway of muscarinic receptor stimulation leading to Egr-1 activation that primarily comprises protein kinase C, MEK1/2, and ERK1/2. Second, the data provide the first evidence that the antibipolar agent sodium valproate substantially attenuates carbachol-stimulated Egr-1 DNA binding activity and that this inhibition occurs through a novel mechanism other than reducing nuclear Egr-1 protein levels. Clearly, the robust Egr-1 activation in response to muscarinic receptor stimulation points to Egr-1 as a significant downstream target of this pathway. Also, the substantial inhibition of muscarinic receptor-stimulated Egr-1 DNA binding activity by therapeutic concentrations of sodium valproate provides an effect of valproate that may be relevant to its therapeutic mechanism of action in bipolar disorder.

## ACKNOWLEDGEMENTS

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## REFERENCES

Altin J. G. A., Kujubu D. A., Raffioni S., Eveleth D. D., Herschman H. R., and Bradshaw R. A. (1991) Differential induction of primary-response (*TIS*) genes in PC12

pheochromocytoma cells and the unresponsive variant PC12nnr5*. *J. Biol. Chem.* **266**, 5401-5406.

Arenander A. T., de Vellis J., and Herschman H. R. (1989) Induction of *c-fos* and *TIS* genes in cultured rat astrocytes by neurotransmitters. *J. Neurosci. Res.* **24**, 107-114.

Asghari V., Wang J. F., Reiach J. S., and Young L. T. (1998) Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. *Mol. Brain. Res.* **58**, 95-102.

Beckmann A. M. and Wilce P. A. (1997) Egr transcription factors in the nervous system. *Neurochem. Int.* **31**, 477-510.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Chen G., Yuan P., Hawver D. B., Potter W. Z., and Manji H. K. (1997) Increase in AP-1 transcription factor DNA binding activity by valproic acid. *Neuropsychopharmacology* **16**, 238-245.

Christy B. and Nathans D. (1989) DNA binding site of the growth factor-inducible protein Zif268. *Proc. Natl. Acad. Sci. USA* **86**, 8737-8741.

Coso O. A., Chiariello M., Kalinec G., Kyriakis J. .M., Woodgett J., and Gutkind J. S. (1995) Transforming G protein-coupled receptors potently activate JNK (SAPK). Evidence for a divergence from the tyrosine kinase signaling pathway. *J. Biol. Chem.* **270**, 5620-5624.

Ebihara T. and Saffen D. (1997) Muscarinic acetylcholine receptor-mediated induction of *zif268* mRNA in PC12D cells requires protein kinase C and the influx of extracellular calcium. *J. Neurochem.* **68**, 1001-1010.

Enslen H. and Soderling T. R. (1994) Roles of calmodulin-dependent protein kinases and phosphatases in calcium-dependent transcription of immediate early genes. *J. Biol. Chem.* **269**, 20872-20877.

Fordyce D. E., Bhat R. V., Baraban J. M., and Wehner J. M. (1994) Genetic and activity-dependent regulation of zif268 expression: association with spatial learning. *Hippocampus* **4**, 559-568.

Gutkind J. S. (1998) Cell growth control by G protein-coupled receptors: from signal transduction to signal integration. *Oncogene* **17**, 1331-1342.

Hodge C., Liao J., Stofega M., Guan K., Carter-Su C., and Schwartz J. (1998) Growth hormone stimulates phosphorylation and activation of Elk-1 and expression of c-fos, egr-1, and junB through activation of extracellular signal-related kinases 1 and 2. *J. Biol. Chem.* **273**, 31327-31336.

Hughes P. and Dragunow M. (1994) Activation of pirenzepine-sensitive muscarinic receptors induces a specific pattern of immediate-early gene expression in rat brain neurons. *Mol. Brain Res.* **24**, 166-178.

Humblot N., Esteve L., Burgun C., Aunis D., and Zwiller J. (1997) 5-Hydroxytryptamine induces TIS8/egr-1 and c-fos expression in PC12 cells: involvement of tyrosine protein phosphorylation. *Eur. J. Neurosci.* **9**, 84-92.

Jope R. S. (1999) Anti-bipolar therapy: mechanism of action of lithium. *Mol. Psychiatry* **4**, 117-128.

Jope R. S. and Song L. (1997) AP-1 and NFκB stimulated by carbachol in human neuroblastoma SH-SY5Y cells are differentially sensitive to inhibition by lithium. *Mol. Brain Res.* **50**, 171-180.

Katayama N., Iwata E., Sakurai H., Tsuchiya T., and Tsuda M. (1993) Additive induction of Egr-1 (*zif268*) mRNA expression in neuroblastoma X glioma hybrid NG108-15 cells via cholinergic muscarinic, $_{\alpha}$-adrenergic, and bradykinin receptors. *J. Neurochem.* **60**, 902-907.

Lim C. P., Jain N., and Cao X. (1998) Stress-induced immediate-early gene, *egr-1*, involved activation of p38/JNK1. *Oncogene* **16**, 2915-2926.

Mitchell F. M., Russell M., and Johnson G. L. (1995) Differential calcium dependence in the activation of c-Jun kinase and mitogen-activated protein kinase by muscarinic receptors in rat 1a cells. *Biochem. J.* **309**, 381-384.

O'Donovan K. J., Tourtellotte W. G., Milbrandt J., and Baraban J. M. (1999) The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci.* **22**, 167-173.

Ozaki N. and Chuang D. M. (1997) Lithium increases transcription factor binding to AP-1 and cyclic AMP response element in cultured neurons and rat brain. *J. Neurochem.* **69**, 2336-2344.

Pacheco M. A. and Jope R. S. (1996) Phosphoinositide signaling in human brain. *Prog. Neurobiol.* **50**, 255-73.

Pacheco M. A. and Jope R. S. (1998) Bipolar therapeutic agents modulate carbachol-stimulated AP-1 activation. *Soc. Neurosci. Abstr.* **24**, 997.

Reiser C. O. A., Lanz T., Hofmann F., Hofer G., and Rupprecht H. D., Goppelt-Struebbe M. (1998) Lysophosphatidic acid-mediated signal-transduction pathways involved in the induction of the early-response genes prostaglandin G/H synthase-2 and Egr-1: a critical role for the mitogen-activated protein kinase p38 and for Rho proteins. *Biochem. J.* **330**, 1107-1114.

Schaefer A., Magocsi M., Fandrich A., and Marquardt H. (1998) Stimulation of the $Ca^{2+}$-mediated egr-1 and c-fos expression in murine erythroleukaemia cells by cyclosporin A. *Biochem. J.* **335**, 505-511.

Unlap T. M. and Jope R. S. (1997) Lithium attenuates nerve growth factor-induced activation of AP-1 DNA binding activity in PC12 cells. *Neuropsychopharmacology* **17**, 12-17.

von der Kammer H., Mayhaus M., Albrecht C., Enderich J., Wegner M., and Nitsch R. (1998) Muscarinic acetylcholine receptors activate expression of the Egr gene family of transcription factors. *J. Biol. Chem.* **273**, 14538-14544.

Wang J. F., Asghari V., Rockel C., and Young L. T. (1999) Cyclic AMP response element binding protein phosphorylation and DNA binding is decreased by chronic lithium but not valproate treatment of SH-SY5Y neuroblastoma cells. *Neuroscience* **91**, 771-776.

Williams M. B. and Jope R. S. (1995) Circadian variation in rat brain AP-1 DNA binding activity after cholinergic stimulation: modulation by lithium. *Psychopharmacology* **122**, 363-368.

Worley P. F., Christy, P. A., Nakabeppu Y., Bhat, R. V., Cole, A. J., and Baraban, J. M. (1991) Constitutive expression of zif268 in neocortex is regulated by synaptic activity. *Proc. Natl. Acad. Sci. USA* **88**, 5106-5110.

Worley P. F., Bhat R. V., Baraban J. M., Erickson C. A., McNaughton B. L., and Barnes C. A. (1993) Thresholds for synaptic activation of transcription factors in hippocampus: correlation with long-term enhancement. *J. Neurosci.* **13**, 4776-4786.

Yuan P. X., Chen G., Huang L. D., and Manji H. K. (1998) Lithium stimulates gene expression through the AP-1 transcription factor pathway. *Mol. Brain. Res.* **58**, 225-230.

# THE MULTIFACETED ROLES OF GLYCOGEN SYNTHASE KINASE 3β IN CELLULAR SIGNALING

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## ABSTRACT

Glycogen synthase kinase-3β (GSK3β) is a fascinating enzyme with an astoundingly diverse number of actions in intracellular signaling systems. GSK3β activity is regulated by serine (inhibitory) and tyrosine (stimulatory) phosphorylation, by protein complex formation, and by its intracellular localization. GSK3β phosphorylates and thereby regulates the functions of many metabolic, signaling, and structural proteins. Notable among the signaling proteins regulated by GSK3β are the many transcription factors, including activator protein-1, cyclic AMP response element binding protein, heat shock factor-1, nuclear factor of activated T cells, Myc, β-catenin, CCAAT/enhancer binding protein, and NFκB. Lithium, the primary therapeutic agent for bipolar mood disorder, is a selective inhibitor of GSK3β. This raises the possibility that dysregulation of GSK3β and its inhibition by lithium may contribute to the disorder and its treatment, respectively. GSK3β has been linked to all of the primary abnormalities associated with Alzheimer's disease. These include interactions between GSK3β and components of the plaque-producing amyloid system; the participation of GSK3β in phosphorylating the microtubule-binding protein tau, which may contribute to the formation of neurofibrillary tangles; and interactions of GSK3β with presenilin and other Alzheimer's disease-associated proteins. GSK3β also regulates cell survival, as it facilitates a variety of apoptotic mechanisms, and lithium provides protection from many insults. Thus, GSK3β has a central role regulating neuronal plasticity, gene expression, and cell survival and may be a key component of certain psychiatric and neurodegenerative diseases.

# INTRODUCTION

Glycogen synthase kinase-3β (GSK3β) was named for its ability to phosphorylate, and thereby inactivate, glycogen synthase, a key regulatory process in the synthesis of glycogen. With such an inauspicious beginning and moniker, it has come as some surprise to find that GSK3β is a critical central figure in many cellular signaling pathways. Now it is known that GSK3β is an important component of signaling systems coupled to receptors for insulin, a variety of growth factors and neurotrophins, and other signaling molecules. However, GSK3β is not only a key component of these signaling systems, but GSK3β is also a critically important regulator of several transcription factors which, in turn, can affect the control of the expression of numerous genes. Furthermore, the control of GSK3β activity is an important component in the regulation of complex functions, encompassing a wide range from survival at the cellular level to mood and cognition at the organism level. These actions, along with further evidence, have implicated the involvement of dysregulated GSK3β activity in certain psychiatric diseases, such as bipolar mood disorder, and neurodegenerative diseases, such as Alzheimer's disease. Thus, in spite of its inauspicious name, GSK3β is a fascinating enzyme that plays crucial roles in many major signaling processes that are involved in key functions of the brain and are associated with dysfunction in some major diseases of the central nervous system. This review focuses on recent developments in the understanding of GSK3β with an emphasis on processes likely to be important to neuronal function.

## GSK3β

There are two highly homologous forms of mammalian GSK3, GSK3α and GSK3β (Woodgett, 1990). GSK3β, the smaller of the two proteins, consists of 482 amino acids with a molecular weight of 46,712 Da and contains a central protein kinase catalytic domain (Woodgett, 1991). An elegant historical synopsis of the discovery, cloning, and characterization of GSK3β is found in a review by Plyte et al. (1992). These authors also reported that GSK3β is ubiquitous throughout the animal kingdom (Plyte et al., 1992). Although GSK-3β was originally isolated from skeletal muscle (Embi et al., 1980; Rylatt et al., 1980), the enzyme is widely expressed in all tissues, with particularly abundant levels in brain (Woodgett, 1990). Leroy and Brion (1999) observed that in the developing rat brain, GSK3β is particularly abundant in neurons relative to astrocytes and that GSK3β levels decrease significantly after postnatal day 20, which correlates temporally with the completion of dendrite extension and synapse formation. Furthermore, the same study showed widespread expression of GSK3β in the adult brain, suggesting a fundamental role for GSK3β in neuronal signaling pathways (Leroy and Brion, 1999). The high degree of conservation of GSK3β throughout evolution, taken together with the abundant expression of GSK3β in brain, denote a fundamental role for GSK3β in intracellular neuronal signaling systems, and further studies implicate altered GSK3β function in neuronal dysfunction and disease, concepts that are discussed further in this review.

An intriguing characteristic of GSK3β involves its requirements for substrate recognition. There is not a strict consensus motif for substrate phosphorylation by

GSK3β, but many GSK3β substrates require prior phosphorylation by a priming kinase to form the motif *-S-X-X-X-S(P)-* before phosphorylation by GSK3β is possible. For these target proteins, phosphorylation by GSK3β is considered hierarchical, in that GSK3β uses a phosphate as part of its recognition requirement (DePaoli-Roach, 1984; Fiol et al., 1988). One example of this hierarchical phosphorylation is the phosphorylation of glycogen synthase by GSK3β (reviewed in Roach, 1990). Dephosphorylated glycogen synthase is not a substrate for GSK3β until it is first phosphorylated by casein kinase II. This primary phosphorylation forms a recognition site for GSK3β, which then phosphorylates four serine residues on glycogen synthase, inhibiting its activity (DePaoli-Roach, 1984; Fiol et al., 1988; Roach, 1990). Hierarchical phosphorylation provides complex regulation at multiple levels because at least two kinases are involved and sequentially ordered phosphorylation is necessary. However, not all substrates require prior phosphorylation for recognition by GSK3β, including members of the myc family of transcription factors. This category of substrates are thought to contain multiple acidic residues that are recognized by GSK3β, thus negating the necessity of phosphorylated amino acids (Fiol et al., 1988).

## REGULATION OF GSK3β

GSK3β is subject to multiple regulatory mechanisms. Although phosphorylation is the most widely studied mechanism of GSK3β regulation, protein complex formation, intracellular localization, and mood-stabilizing drugs have also been shown to influence GSK3β activity. Such complex regulatory mechanisms are necessary for an enzyme that modifies multiple and diverse substrates, including metabolic, signaling, and structural

proteins and transcription factors, and influences consequential cellular processes such as gene expression and cell viability.

*Inactivation of GSK3β by serine phosphorylation*

The activity of GSK3β can be reduced by phosphorylation of Serine-9 (reviewed in Plyte et al., 1992). Several kinases have been found to be capable of mediating this modification, including p70 S6 kinase, p90Rsk (also called MAPKAP kinase-1), Akt (also called protein kinase B), certain isoforms of protein kinase C, and cyclic AMP-dependent protein kinase (protein kinase A). Much research has been directed toward identifying which receptor-coupled signaling systems use each of these kinases to control GSK3β activity. Many of these interactions are summarized in Fig. 1. As discussed in later sections of this article, the inhibitory control of GSK3β by serine-9 phosphorylation is a critical factor in many receptor-coupled signaling processes, in the antiapoptotic actions of the Akt signaling pathway, and in certain diseases.

In the first investigations of GSK3β phosphorylation, Sutherland et al. (1993) reported that p90Rsk and p70 S6 kinase could phosphorylate and inactivate GSK3β in vitro. Furthermore, they reported that GSK3β inhibited by phosphorylation could be re-activated with protein phosphatase 2A (Sutherland et al., 1993). Further investigations have substantiated the regulatory role of p90Rsk in controlling GSK3β activity, but the influence of p70 S6 kinase remains unclear. Stambolic and Woodgett (1994) found that GSK3β expressed in HeLa cells was phosphorylated on serine-9 by activation of p90Rsk, and Saito et al. (1994) also concluded that p90Rsk, but not p70 S6 kinase or protein

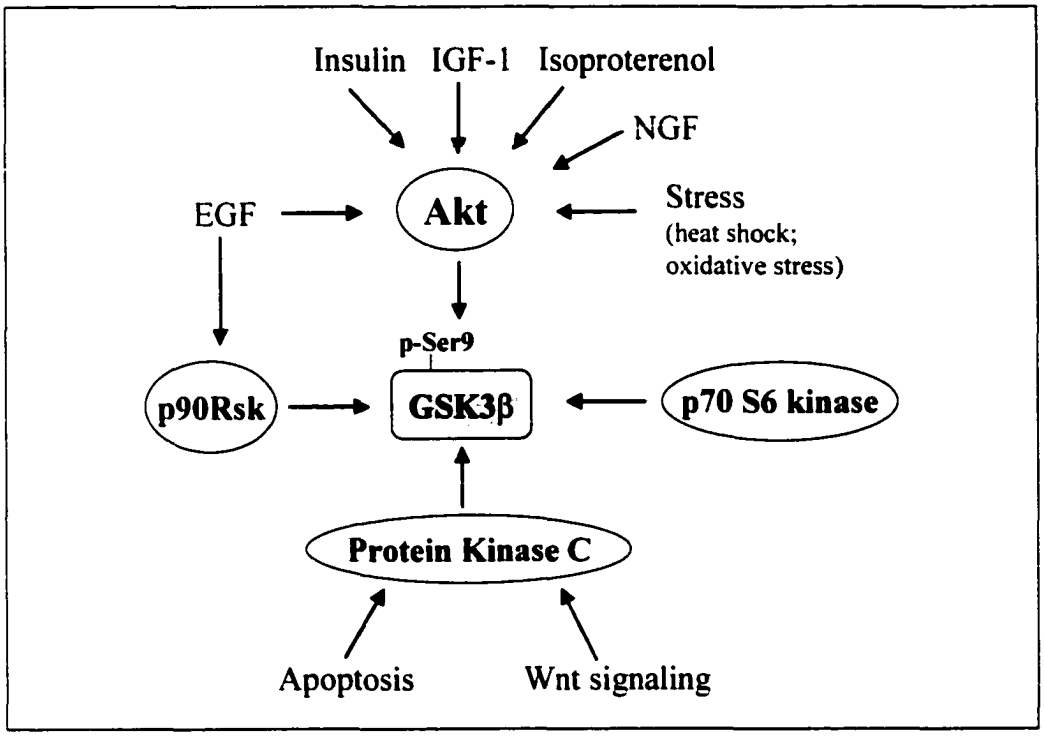


**FIG. 1.** Phosphorylation-dependent inhibition of GSK3β. Phosphorylation of serine-9 of GSK3β inhibits its activity. Some of the kinases able to phosphorylate this site on GSK3β, and stimuli leading to activation of those kinases, are depicted.

kinase C, was responsible for the phosphorylation of GSK3β induced by epidermal growth factor (EGF) in human epidermoid A431 cells.

Insulin treatment inactivates GSK3β, an effect that is associated with serine-9 phosphorylation of GSK3β, and Akt was identified as the major kinase mediating this response to insulin (Cross et al., 1995), as summarized in Fig. 2. Akt is activated by a signaling cascade involving phosphatidylinositol 3-kinase (PI3K), 3-phosphoinositide-dependent kinase-1, and probably other kinases as well, notably integrin-linked kinase, which may also directly phosphorylate GSK3β (Delcommenne et al., 1998). Stimulation of certain receptors, such as the insulin receptor, activates this signaling cascade, leading to activation of Akt, which is associated with increased phosphorylation of serine-473 and threonine-308 of Akt (Alessi et al., 1996). After the seminal findings of Cross et al. (1995), the insulin-induced inactivation of GSK3β in myoblasts (Hurel et al., 1996) and rat epididymal fat cells (Moule et al., 1997) also was found to be associated with activation of Akt. Shaw et al. (1997) reported that Akt could phosphorylate serine-9 of GSK3β in intact cells and concluded that this was the mechanism by which insulin inhibits the activity of GSK3β. van Weeren et al. (1998) extended the finding that Akt phosphorylates and inactivates GSK3β after insulin treatment by also showing a direct interaction between the two proteins, indicating that this is a direct, not indirect, regulation of GSK3β by Akt. Overall, substantial evidence supports the conclusion that insulin causes inhibition of GSK3β and that this is mediated by direct phosphorylation by Akt.

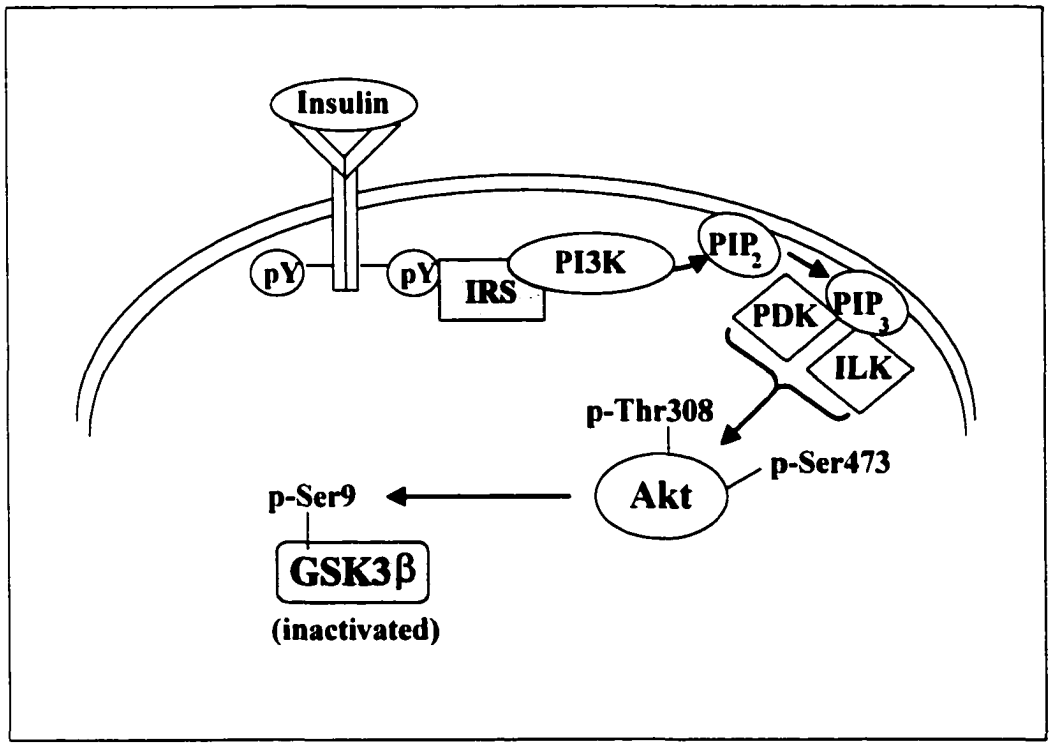


**FIG. 2.** Scheme of pathway mediating insulin-induced inactivation of GSK3β. Insulin initiates a signaling cascade that results in Akt-induced phosphorylation and inactivation of GSK3β.

Several other stimuli have been demonstrated to cause inhibition of GSK3β, with some indications that Akt mediates this effect. Insulin-like growth factor-1 (IGF-1) inhibits GSK3β through increased serine-9 phosphorylation in a PI3K-dependent manner in cultured cerebellar granule cells (Cui et al., 1998) and primary cultured neurons (Quevedo et al., 2000), thus resembling the signaling induced by insulin. However, in hepatocytes, Park et al. (1999) noted differences in the signaling induced by insulin and by IGF-1 that leads to inhibition of GSK3β, as both activated Akt to the same extent but IGF-1 caused less inhibition of GSK3β. Thus, Park et al. (1999) concluded that activation of Akt is necessary, but not sufficient, to inactivate GSK3β. Nerve growth factor treatment of PC12 cells also inhibited GSK3β activity through a PI3K-dependent mechanism, implicating Akt as the mediating kinase (Pap and Cooper, 1998). Endothelin-1 inhibited GSK3β through serine-9 phosphorylation in a PI3K-dependent manner in myocytes (Haq et al., 2000). Activation of Akt and inhibition of GSK3 was also caused by EGF treatment of muscle cells (Halse et al., 1999) as opposed to the signaling activated by EGF in NIH 3T3 cells that involves p90Rsk (Eldar-Finkelman et al., 1995), which led Halse et al. (1999) to discuss a variety of mechanisms that may account for the different responses induced by EGF in different cell systems. In rat epididymal fat cells, isoproterenol acting primarily through β3-adrenoreceptors activated Akt and inhibited GSK3 activity, but these responses were insensitive to inhibitors of PI3K, indicating that another mechanism for activating Akt was used (Moule et al., 1997). Isoproterenol-stimulated β-adrenergic receptors also increased serine-9 phosphorylation of GSK3β, and reduced its activity, in myocytes (Morisco et al., 2000). Thus, a variety of reports indicate

that other stimuli in addition to insulin also utilize Akt to inhibit GSK3β, but PI3K-dependent and -independent mechanisms appear to couple different receptors to Akt.

Cell stressors can modulate the activity of GSK3β, as both heat shock and oxidative stress can cause inhibition of GSK3β. Some studies (Lin et al., 1997; Shaw et al., 1998; Bijur and Jope, 2000), but not all (Konishi et al., 1996), indicated that heat shock activates Akt through PI3K activation, and the PI3K-dependent Akt activation has been linked to inhibition of GSK3β (Shaw et al., 1998). However, in HeLa cells, heat shock did not lower GSK3β activity, but there was a dramatic rise in GSK3β activity during recovery at 37°C following transient heat shock through an unknown mechanism (He et al., 1998). Xavier et al. (2000) also observed heat shock-induced activation of GSK3β in Xenopus oocytes. $H_2O_2$ was reported to reduce the activity of GSK3β in a PI3K-dependent manner, which implicates Akt as the mediating kinase (Blair et al., 1999). Exposure of cells to the neurotoxic Alzheimer's disease-associated Aβ peptide has been reported to increase GSK3β-mediated phosphorylation (Takashima et al., 1995, 1998a), apparently due to an inhibitory effect of Aβ on PI3K leading to activation of GSK3β (Takashima et al., 1996). Stress-induced regulation of GSK3β appears to be an important mechanism in cellular signaling necessary for survival from potentially lethal insults but much still remains to be learned about this potentially important cellular response to stress.

In addition to p90Rsk and Akt, protein kinase A, and certain isoforms of protein kinase C also increase serine-9 phosphorylation of GSK3β, resulting in inhibition of activity. Fang et al. (2000) recently found that activation of protein kinase A directly phosphorylated serine-9 of GSK3β. Goode et al. (1992) reported that several isoforms of

protein kinase C (α, βI, βII, γ, not ε) phosphorylated and inactivated GSK3β. Protein kinase C has been implicated in wnt/Wingless signaling-induced inhibition of GSK3β (Cook et al., 1996). Tsujio et al. (2000) made the interesting observation that apoptosis-linked partial proteolysis of protein kinase C-δ produced an active fragment that was capable of phosphorylating and inhibiting GSK3β. Thus, although protein kinase C is potentially a major regulator of GSK3β, this action has not yet been thoroughly investigated.

Taken together, these investigations reveal that there are multiple mechanisms that can regulate the inactivation of GSK3β via serine-9 phosphorylation. Although the PI3K/Akt pathway may predominate in inhibiting GSK3β in many situations, it appears that different signaling mechanisms regulate the inactivation of GSK3β dependent on both the type of receptor activated or stimulus applied, and on the cell type. Much still remains to be learned about the systems that inhibit GSK3β by serine-9 phosphorylation, and, notably, very little is known about the reactivation of GSK3β by phosphatase-mediated dephosphorylation of serine-9.

*Activation of GSK3β by tyrosine phosphorylation*

In opposition to the inhibitory modulation of GSK3β that occurs by serine phosphorylation, tyrosine phosphorylation of GSK3β increases the enzyme's activity, as depicted in Fig. 3. Hughes et al. (1993) found that GSK3β in vivo is tyrosine phosphorylated and that its activity is dependent on tyrosine phosphorylation on tyrosine-216. This conclusion was supported by Wang et al. (1994a), who also provided evidence for autophosphorylation of this site. Murai et al. (1996) found that the tyrosine

phosphorylation, and thus activity, of GSK3β is regulated by intracellular signaling systems. Treatment of cells with insulin or EGF, or direct activation of protein kinase C with a phorbol ester, decreased both the activity and tyrosine phosphorylation of GSK3β, suggesting that these agents stimulated the action of a phosphotyrosine phosphatase acting on GSK3β (Murai et al., 1996). However, these results were contradicted by Shaw et al. (1997), who found that the insulin-induced decrease in GSK3β activity was solely due to increased serine-9 phosphorylation and not to decreased tyrosine phosphorylation.

Several recent studies have begun to clarify further the cellular signaling systems that affect GSK3β activity by regulating its tyrosine phosphorylation. In Dictyostelium, Kim et al. (1999) reported that the tyrosine kinase ZAK1 increases GSK3β activity, likely as the result of direct tyrosine phosphorylation. Lesort et al. (1999b) reported the novel finding that Fyn, a member of the src tyrosine kinase family, may directly tyrosine phosphorylate, and thereby activate, GSK3β. The tyrosine phosphorylation of GSK3β also was recently found to be increased by transient, physiologically relevant, increases in intracellular calcium concentrations (Hartigan and Johnson, 1999). Several proapoptotic stimuli recently were found to increase the activity and tyrosine-216 phosphorylation of GSK3β (Bhat et al., 2000), but the kinase mediating this modification was not investigated.

Overall, although studies of the tyrosine phosphorylation of GSK3β are relatively sparse, it is evident that this plays an important role in regulating the activity of GSK3β. In particular, the recent revelations that a member of the src tyrosine kinase family (Lesort et al., 1999b) and that calcium transients (Hartigan and Johnson, 1999) participate

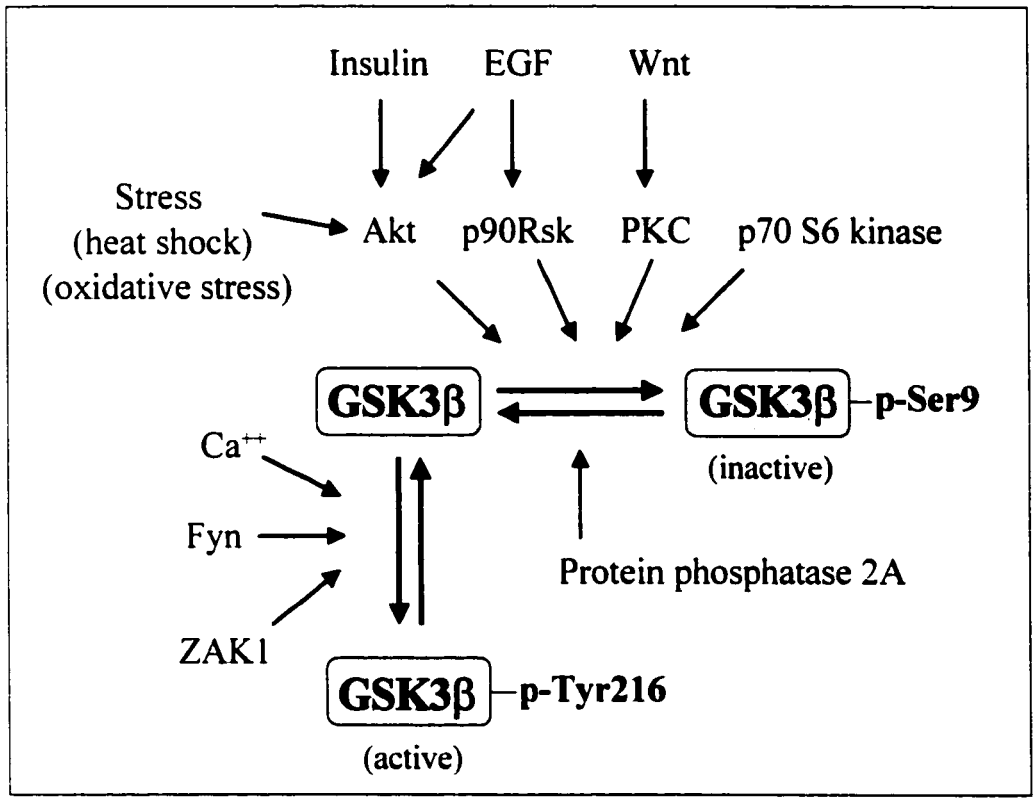


**FIG. 3.** Phosphorylation-dependent regulation of GSK3β. Some of the signals mediating the activation of GSK3β via phosphorylation of tyrosine-216 and the inhibition of GSK3β via phosphorylation of serine-9 are depicted.

in regulating the tyrosine phosphorylation of GSK3β indicate that this modulatory mechanism may participate in many intracellular signaling cascades that involve these regulators.

*Regulation of GSK3β by protein complex formation and intracellular localization*

One of the newer mechanisms that has been found to be important in the regulation of GSK3β activity and is currently under intense scrutiny is modulation by protein complex formation mediated by GSK3β binding proteins. These include such proteins as GSK3β binding protein (GBP) (Yost et al., 1998), Axin (Ikeda et al., 1998; Yamamoto et al., 1999) and the Axin-related protein named both Axil (Yamamoto et al., 1998) and Conductin (Behrens et al., 1998). Interactions of these proteins with GSK3β are depicted in Fig. 4.

GBP was discovered in a two-hybrid screen for GSK3β-binding proteins in *Xenopus* (Yost et al., 1998). GBP represents the prototype of a family of GSK3β-binding proteins, which presently is known to include Frat 1 and 2 (Jonkers et al., 1997; Li et al., 1999; Thomas et al., 1999), which inhibit GSK3β activity by forming protein complexes (Yost et al., 1998). This mechanism was shown to allow local inhibition of GSK3β, leading to stabilization of β-catenin levels due to reduced GSK3β-facilitated degradation of β-catenin, a necessary step in embryo development (Yost et al., 1998; Farr et al., 2000). An important property of GSK3β bound to GBP was also revealed by the finding that GBP did not inhibit the catalytic activity of GSK3β, leaving it able to phosphorylate a peptide substrate, but GBP binding inhibited GSK3β-mediated phosphorylation of the

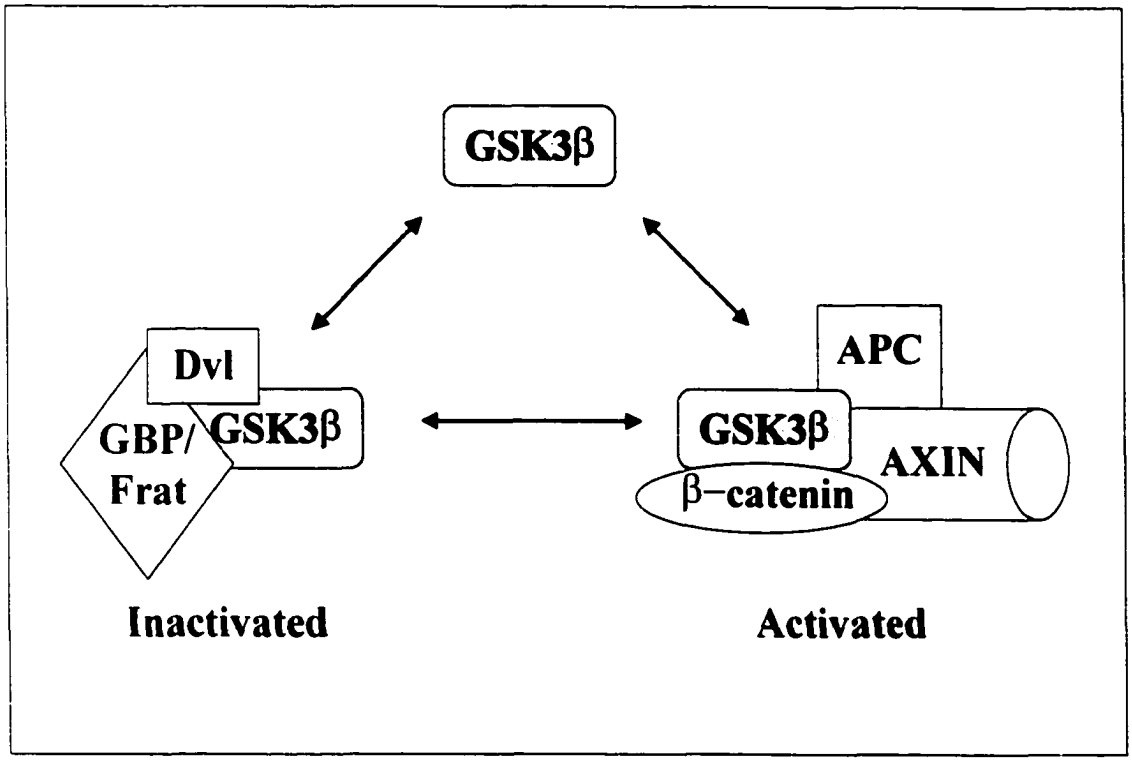


**FIG. 4.** Regulation of GSK3β by protein complex formation. Axin acts as a scaffold bringing together GSK3β with substrates such as β-catenin and APC. GBP and Frat 1 and 2 bind GSK3β to inhibit its activity, a process facilitated by Dvl.

intact tau protein, possibly due to steric hindrance, indicating that the widespread use of peptide substrates to measure the activity of GSK3β may not detect the actual ability of GSK3β to phosphorylate intact proteins (Farr et al., 2000). Additional evidence indicates that binding of GBP family members to GSK3β limits the phosphorylating activity of GSK3β toward only some, not all, substrates, and the intriguing idea was proposed that the inhibition was primarily of substrates that do not require a priming pre-phosphorylation event necessary for GSK3β-mediated phosphorylation (Thomas et al., 1999). Overall, it is apparent from these initial studies that complex formation between GSK3β and proteins in the GBP family is an important mechanism for the inhibitory control of GSK3β activity, and further studies in this newly emerging field will surely reveal further intricacies about this relationship.

Opposite to the inhibitory effect of complex formation with GBP, GSK3β activity is enhanced by binding to Axin, which is both a substrate for phosphorylation by GSK3β and enhances GSK3β-induced phosphorylation of β-catenin (Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Kishida et al., 1998; Nakamura et al., 1998; Sakanaka et al., 1998; Hedgepeth et al., 1999; Yamamoto et al., 1999). These studies demonstrated that Axin, the adenomatous polyposis coli gene product (APC), GSK3β, and β-catenin form a tetrameric complex (to which phosphatases can also bind) that regulates the stabilization of β-catenin (Rubinfeld et al., 1996; Kishida et al., 1998). Phosphorylation by GSK3β of both Axin and APC may facilitate formation of this complex, which then increases the phosphorylation of β-catenin to enhance its degradation (Rubinfeld et al., 1996; Hedgepeth et al., 1999; Farr et al., 2000). Facilitation of this complex formation may be due in part to the finding that GSK3β-mediated phosphorylation of Axin stabilizes Axin

from degradation, whereas inhibition of GSK3β by lithium causes a reduction of Axin levels (Yamamoto et al., 1999). An Axin-like protein, known both as Axil and as Conductin, appears to function similarly to Axin by facilitating GSK3β-mediated phosphorylation of β-catenin (Yamamoto et al., 1998; Behrens et al., 1998).

There is also evidence of multiprotein complex formation based on a scaffold of presenilin 1 (Czech et al., 2000). Mutations of presenilin 1 can cause familial Alzheimer's disease, so the functions of this protein are currently under intense scrutiny. Among the recent findings, presenilin 1 was found to bind both GSK3β and β-catenin and, in doing so, to regulate GSK3β-mediated phosphorylation of β-catenin (Takashima et al., 1998b; Kang et al., 1999; Gantier et al., 2000). These interactions raise the possibility that multiple GSK3β binding proteins, in addition to those related to GBP and Axin, contribute to regulating GSK3β activity.

Thus, it now appears that GBP and Axin, and likely additional proteins, have prominent roles in regulating the activity of GSK3β through protein complex formation. Recent evidence supports the view that GBP and Axin cannot bind GSK3β simultaneously, suggesting a competitive mechanism for protein complex formation that regulates GSK3β activity (Farr et al., 2000). However, much remains to be learned about processes regulating these protein complexes. One such regulatory mechanism was recently revealed by the discovery that Dvl can bind both Axin and Frat 1 and may reduce GSK3β activity by facilitating the binding of Frat 1 to GSK3β and enhancing the dissociation of GSK3β from Axin (Li et al., 1999; Krylova et al., 2000). Additionally, a new Axin-binding protein, named Axam, was recently identified and inhibits complex formation of Dvl with Axin and the inhibitory effect of Dishevelled on GSK3β activity

(Kadoya et al., 2000). Further recent evidence indicates that free GSK3β and GSK3β bound to Axin participate in independent signaling pathways (Ding et al., 2000). Overall, these exciting new findings have revealed that, in addition to serine and tyrosine phosphorylation causing inhibition and activation of GSK3β activity, respectively, binding of GSK3β to GBP family members and to Axin family members also causes inhibition and activation of GSK3β activity, respectively. Thus, GSK3β activity is regulated both by phosphorylation and by protein complex formation, and future investigations should reveal how these regulatory mechanisms are integrated to provide precise control of GSK3β activity and may reveal mechanisms for substrate-specific regulation of the action of GSK3β.

*Regulation of GSK3β by subcellular localization*

Another emerging topic in the study of the regulation of GSK3β concerns its subcellular localization. Some substrates of GSK3β, such as tau, are cytosolic, whereas others, notably several transcription factors, are nuclear. Thus, it is evident that GSK3β must be located in both compartments, but surprisingly little is known about the regulation of the intracellular localization of GSK3β. Ragano-Caracciolo et al. (1998) reported that GSK3β is enriched in a glycogen fraction associated with the nuclear envelope. Cell cycle-dependent changes in the intracellular localization of GSK3β were detected by Diehl et al. (1998), who found increased nuclear GSK3β during the S phase in NIH3T3 cells. Increased nuclear GSK3β was induced by heat shock (Xavier et al., 2000) and by endothelin-1 in myocytes (Haq et al., 2000). Bhat et al. (2000) recently

reported that proapoptotic stimuli increased tyrosine-216-phosphorylated GSK3β in the nucleus, but it remains to be determined if this represents translocation of GSK3β or increased tyrosine phosphorylation of GSK3β already in the nucleus.

The dual compartmentalization of GSK3β raises many questions about the mechanisms regulating its activity. Which kinases and phosphatases acting on GSK3β are in each compartment? Which of the GSK3β-binding proteins are in the nucleus as well as in the cytosol? Is transport of GSK3β in and out of the nucleus a dynamic process, and, if so, how is it regulated? Which compartment of GSK3β is most critical for each of GSK3β's actions, both at the protein level (which proteins are phosphorylated by GSK3β in each compartment) and at the functional level (which compartment of GSK3β is responsible for its effects on cell survival and which are most important for the actions of lithium)? Integrating the mechanisms regulating the phosphorylation, protein complex formation, and intracellular localization of GSK3β remains a challenge for future investigations in order to more fully understand how GSK3β is regulated and how it regulates many cellular functions.

*Regulation of GSK3β by mood-stabilizing and other drugs*

The identification of a specific inhibitor for any enzyme provides an exceptionally valuable investigative tool. Such has been the case with the relatively recent discovery that lithium is a selective inhibitor of GSK3β (Klein and Melton, 1996). Although lithium is not a specific inhibitor of GSK3β since it also inhibits other enzymes, such as inositol phosphatases involved in the phosphoinositide signal transduction system, GSK3β is the

only kinase known to be inhibited by lithium, with the exception of the closely related GSK3α, a degree of selectivity that has proved valuable in many studies (reviewed in Jope, 1999a). Additionally, not only has this discovery been of immense use in clarifying functions of GSK3β, but because lithium is a therapeutic drug used for the treatment of bipolar mood disorder, the discovery of this site of action of lithium has been a stimulus for studies of the therapeutic mechanism of action of lithium and of the related question of the biochemical abnormalities underlying mood disorders. Finally, the important effects of lithium that can be attributed to inhibition of GSK3β have added impetus for the development of new drugs that inhibit GSK3β.

The discovery that lithium inhibits GSK3β was based on remarkably insightful observations of lithium's effects on *Xenopus* development (Klein and Melton, 1996). These investigators noted that dorsalization caused by lithium treatment was similar to that associated with ectopic expression of *wnt* genes in *Xenopus* embryos, which is known to cause activation of signaling leading to inhibition of GSK3β (Klein and Melton, 1996). Experiments with purified GSK3β confirmed the in vivo observations that lithium inhibited actions of GSK3β and demonstrated that lithium directly inhibits the enzyme with an $IC_{50}$ of approximately 2 m*M* (Klein and Melton, 1996). Klein and Melton (1996) also demonstrated for the first time that lithium inhibited the GSK3β-mediated phosphorylation of tau protein and of protein phosphatase inhibitor-2, and that lithium did not inhibit several protein kinases other than GSK3β. Shortly thereafter, Stambolic et al. (1996) also reported lithium's selective inhibitory influence on GSK3β, as well as on GSK3α, and further showed that lithium was an effective inhibitor of GSK3β in intact cells by assessing its inhibitory effect on GSK3β-mediated phosphorylation of tau.

Through its inhibition of GSK3, lithium was also found to increase cytoplasmic levels of β-catenin and Armadillo, proteins that are vulnerable to enhanced degradation as a result of GSK3-mediated phosphorylation (Stambolic et al., 1996).

These seminal studies provided the basis for many investigations that have added to the understanding of GSK3β in cell signaling and the mechanisms of action of lithium, many of which have focused on β-catenin. Phosphorylation of β-catenin by GSK3β enhances the degradation of β-catenin, and several studies have found that lithium treatment increases β-catenin levels (Stambolic et al., 1996; Hedgepeth et al., 1997; Orford et al., 1997). However, lithium was also reported to activate β-catenin signaling by a mechanism other than modulation of β-catenin levels through regulation of proteosomal degradation, raising the intriguing possibility that lithium has additional sites of action influencing β-catenin signaling (Nelson and Gumbiner, 1999). These studies demonstrate that lithium activates the Wnt signaling pathway, which normally involves inhibition of GSK3, an action that may account for numerous observations of developmental effects of lithium (Hedgepeth et al., 1997). Since phosphorylation of Axin by GSK3β enhances the degradation of Axin, lithium treatment resulted in increased levels of Axin (Yamamoto et al., 1999). Because tau protein, a microtubule-associated protein, is an established target of phosphorylation by GSK3β and because site-specific phosphorylation-dependent tau antibodies are available, several studies have used tau to asses the inhibitory effects of lithium on GSK3β with consistent findings that lithium reduced tau phosphorylation in a wide variety of cell systems (Klein and Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997). Additional studies using lithium to inhibit GSK3β to alter the phosphorylation status of target proteins are discussed in relevant

sections later in this article. Especially noteworthy are those studies assessing the phosphorylation state of transcription factors which may be especially relevant in the therapeutic mechanism of action of lithium in bipolar disorder.

The potential importance of GSK3β in the neuropathology of several diseases has led to much current interest in identifying selective inhibitors of GSK3β that may be therapeutically useful, as indicated by a flurry of recent patent applications (Castro and Martinez, 2000). Some interesting leads of new selective inhibitors have begun to emerge. Recent reports have indicated that certain derivatives of paullones (Leost et al., 2000), maleimide (Coghlan et al., 2000), and of indirubin (Leclerc et al., 2001) caused selective and potent inhibition of GSK3β. Thus, it appears that important new tools soon may be available for discriminating specific effects of GSK3β in studies of signaling systems, and new therapeutic agents may be developed based on selective inhibition of GSK3β.

*Wnt signaling*

GSK3β is a key component of the Wnt signaling pathway in vertebrates and the highly homologous wingless signaling pathway in *Drosophila*. Each of these signaling pathways has been discussed in detail in several recent excellent reviews (Dale, 1998; Wodarz, 1998; Patapoutian and Reichardt, 2000; Peifer and Polakis, 2000; Seidensticker and Behrens, 2000); so, only a very brief discussion of Wnt signaling is included here to highlight the participation of GSK3β.

Wnt refers to a large family of secreted glycoproteins that signal by activating the Frizzled family of membrane-bound receptors. This Wnt signaling pathway is an integral

component of embryonic development, including central nervous system development, and also appears to function in adults (McMahon and Bradley, 1990; Patapoutian and Reichardt, 2000). In the absence of a Wnt ligand, GSK3β, in concert with the scaffolding protein Axin, phosphorylates β-catenin, the primary downstream effector of Wnt signaling, targeting it for ubiquitination and proteasomal degradation (Fig. 5) (Hart et al., 1998; Peifer and Polakis, 2000). The binding of a Wnt ligand to the extracellular domain of the seven-transmembrane-spanning receptor Frizzled activates the Wnt signaling cascade. Although all of the regulatory mechanisms are not known, this signaling activity causes inactivation of GSK3β, which leads to increased cytoplasmic β-catenin levels and translocation of β-catenin to the nucleus where, acting with Tcf-Lef proteins, the transcription of Wnt target genes is activated (Behrens et al., 1996; Dale, 1998). Thus, GSK3β must be inactivated for Wnt signaling to proceed, and the presence of active GSK3β negatively regulates Wnt signaling.

Protein-protein interactions and protein phosphorylation have both been implicated in the mechanism of Wnt-mediated inactivation of GSK3β, which are summarized in Fig. 5. Multiple studies have demonstrated that activation of Wnt signaling results in phosphorylation of the cytoplasmic adaptor protein Dishevelled, which recruits the inhibitory GSK3β-binding-protein Frat1 (Li et al., 1999). Frat1 binds and inactivates GSK3β, blocking phosphorylation of β-catenin (Thomas et al., 1999; Polakis, 2000). Additional studies have shown that activated Dishevelled binds and sequesters Axin, decreasing the susceptibility of β-catenin to phosphorylation by GSK3β (Li et al., 1999; Seidensticker and Behrens, 2000). Additionally, protein kinase C activity is required for Wnt-mediated inhibition of GSK3β, suggesting that protein kinase C may

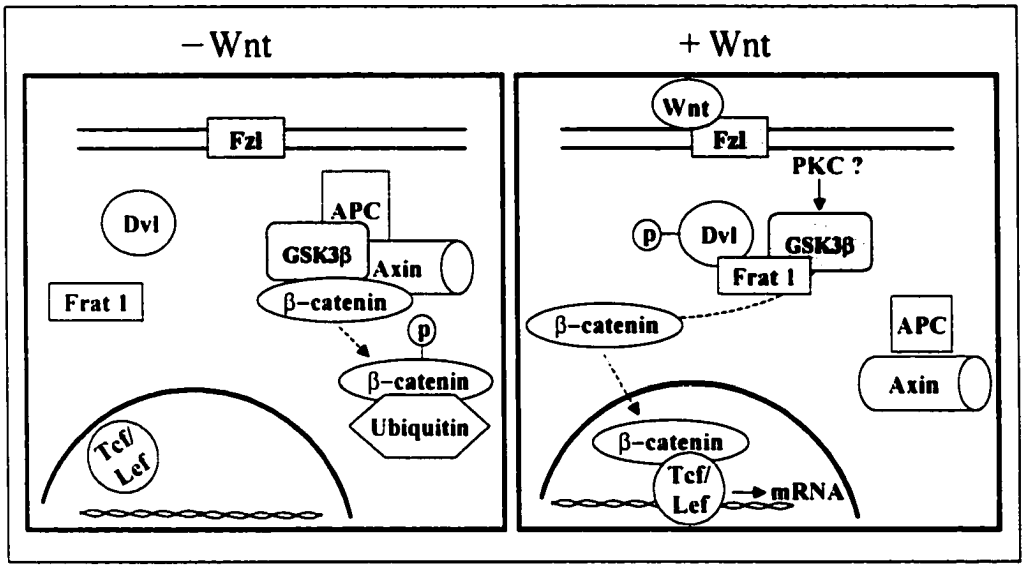


**FIG. 5.** Regulation of GSK3β by Wnt signaling. In the absence of Wnt, GSK3β is complexed with Axin and APC and phosphorylates β-catenin, targeting it for degradation. Wnt activates the Fzl receptor, resulting in Dvl and Frat 1 binding to GSK3β, inhibiting its activity, a process possibly facilitated by protein kinase C. Inhibition of GSK3β results in increased β-catenin levels, its translocation to the nucleus, and facilitation of Tcf-Lef-mediated transcription.

directly phosphorylate and inhibit GSK3β, preventing its phosphorylation of β-catenin (Cook et al., 1996; Dale, 1998; Chen et al., 2000b). Although the mechanisms remain to be more completely clarified, it is clear that Wnt signaling inhibits that activity of GSK3β and that this regulation of GSK3β integrates both protein complex formation and phosphorylation mechanisms to control the activity of GSK3β.

## WHAT DOES GSK3β REGULATE ?

GSK3β phosphorylates a diverse group of substrates (Table 1), few of which have been studied in enough detail to describe the sites phosphorylated and the ultimate effect on substrate function. The first identified substrate of GSK3β was its namesake, the metabolic enzyme glycogen synthase. Probably the substrate of GSK3β that has been studied in most depth is the structural protein tau, a microtubule-associated protein (MAP) that is directly involved in the neuropathology of Alzheimer's disease. Perhaps the largest individual class of proteins that is modified by GSK3β are the transcription factors.

**Table 1.** Mammalian proteins phosphorylated by GSK3β

| Metabolic and signaling proteins | Structural proteins | Transcription factors |
|---|---|---|
| AcetylCoA carboxylase | Dynamin-like protein | AP-1 (Jun family) |
| Amyloid precursor protein | MAP1B | β-catenin |
| APC | MAP2 | C/EBPα |
| ATP-citrate lyase | Neural cell adhesion protein | CREB |
| Axin | Neurofilaments | HSF-1 |
| Cyclin D1 | Ninein | Myc |
| eIF2B | Tau | NFAT |
| Glycogen synthase | | NFκB |
| Insulin receptor substrate-1 | | |
| Myelin basic protein | | |
| NGF receptor | | |
| Protein kinase A | | |
| Protein phosphatase-1 | | |
| Protein phosphatase inhibitor-2 | | |
| Pyruvate dehydrogenase | | |

*Metabolic and signaling proteins*

GSK3β regulates through phosphorylation the activities of several metabolic enzymes. The first of these discovered was, of course, glycogen synthase, which is inhibited when phosphorylated by GSK3β (Embi et al., 1980). The substantial literature on the regulation of glycogen synthase by GSK3β has been reviewed previously (Plyte et al., 1992). Since that initial discovery, several other enzymes have been found to be phosphorylated by GSK3β. Hemmings et al. (1982a) found that GSK3β phosphorylated the type-II regulatory subunit of cyclic-AMP-dependent protein kinase and that ATP-citrate lyase is phosphorylated by GSK3β (Hemmings et al., 1982b). Protein phosphatase inhibitor-2 is inhibited by GSK3β-mediated phosphorylation, which consequently results in activation of Mg-ATP-dependent protein phosphatase-1 (Hemmings et al., 1982b; Wang et al., 1994b), and phosphorylation of protein phosphatase inhibitor-2 was blocked by the GSK3β inhibitor lithium (Klein and Melton, 1996). The G-component of protein phosphatase-1 (Fiol et al., 1988; Dent et al., 1989), acetylCoA carboxylase (Hughes et al., 1992), nerve growth factor receptor (Taniuchi et al., 1986), and myelin basic protein (Yang, 1986) were also early identified substrates of GSK, prior to separation of the α and β subtypes. Threonine-171 of the rat, but not human, glucocorticoid receptor is phosphorylated by GSK3β, a modification that represses the receptor's transcriptional activity (Rogatsky et al., 1998). Insulin receptor substrate-1 is phosphorylated by GSK3β, which results in attenuation of signaling by the insulin receptor (Eldar-Finkelman and Krebs, 1997). GSK3β phosphorylates threonine-268 of cyclin D1, which regulates the intracellular localization and turnover of cyclin D1 (Diehl et al., 1998). Also both Axin and APC are phosphorylated by GSK3β.

Thus, a diverse group of proteins involved in signaling cascades have been found that are regulated by phosphorylation by GSK3β.

Several interactions between GSK3β and proteins associated with Alzheimer's disease have been discovered. These include the findings that GSK3β phosphorylates threonine-743 of the amyloid precursor protein (Aplin et al., 1996) and increases the cellular maturation of the amyloid precursor protein (Aplin et al., 1997), actions that may alter the production of the Alzheimer's disease-associated Aβ peptide. GSK3β also phosphorylates and inhibits the key mitochondrial enzyme pyruvate dehydrogenase (Hoshi et al., 1996). Besides being an important component of the mitochondrial Krebs cycle, pyruvate dehydrogenase is also a key enzyme providing the precursor acetyl coenzyme A that is necessary for the synthesis of the neurotransmitter acetylcholine (Gibson et al., 1975). Hoshi et al. (1997) further reported that the Aβ peptide suppressed acetylcholine synthesis in association with GSK3β-induced inhibition of pyruvate dehydrogenase. Therefore, inhibition of pyruvate dehydrogenase by GSK3β activity may both reduce energy production and impair the synthesis of acetylcholine, conditions that have been postulated to contribute to selective neuronal dysfunction in Alzheimer's disease (Imahori and Uchida, 1997). These and other findings have heightened interest in clarifying the associations of GSK3β with neuropathological changes occurring in Alzheimer's disease.

GSK3β also regulates the activity of eukaryotic initiation factor 2B (eIF2B), the multimeric guanine-nucleotide-exchange factor that facilitates the conversion of inactive eIF2-GDP to active eIF2-GTP, a critical step required for all translation initiation events (reviewed in Webb and Proud, 1997). Because eIF2B is a key site of regulation for

eukaryotic protein synthesis, eIF2B activity is strictly controlled by multiple mechanisms, one of which is phosphorylation of the ε subunit, the largest of its subunits and the most active component in the GDP/GTP exchange (Fabian et al., 1997). Welsh and Proud (1993) first reported that GSK3β phosphorylated the ε subunit of eIF2B in vitro, and Singh et al. (1996) later demonstrated that this phosphorylation by GSK3β inhibited eIF2B activity and blocked activation of eIF2B by casein kinases I and II. Welsh et al (1998) further reported that GSK3β phosphorylated eIF2B at serine-540, inhibiting its activity, and demonstrated the requirement of a priming kinase to phosphorylate eIF2B at serine-544 to create the phosphorylation site for GSK3β (Welsh et al., 1998). A variety of stimuli that activate eIF2B do so through inactivation of GSK3β, including calcium ionophores, phorbol ester-induced activation of protein kinase C, insulin, and growth factors (Welsh et al., 1996; Kleijn et al., 1998; Quevedo et al., 2000; Kleijn and Proud, 2000). Several groups have reported that insulin- and growth factor-induced inhibition of GSK3β activity and subsequent activation of eIF2B requires activation of the PI3K-Akt cell survival pathway (Welsh et al., 1997; Kleijn et al., 1998; Quevedo et al., 2000). These findings provide an example of how components of cell survival signaling cascades counteract the actions of proapoptotic proteins, such as GSK3β to regulate the activity of critical cellular proteins such as eIF2B.

*Structural proteins*

GSK3β phosphorylates several proteins that contribute to the structural characteristics and dynamics of cells. The most frequently studied of these structural protein substrates of GSK3β are critical components of the cytoskeleton, MAPs, tau

(reviewed by Johnson and Hartigan, 1998), and subtypes of MAP1 and MAP2 (reviewed by Sanchez et al., 2000). Microtubules are one of the key components of the cellular cytoskeleton, which is an important determinant of neuronal morphology, and regulation of cytoskeletal dynamics is critical for neuronal plasticity. The stability and dynamics of microtubules can be regulated by MAPs, and, in turn, their functions are regulated by phosphorylation and dephosphorylation.

Tau binds to microtubules in a phosphorylation-dependent manner, and this interaction contributes to the stability of microtubules and, thus, to neuronal structure and to structural changes associated with neuronal plasticity (Johnson and Hartigan, 1998). Tau has been a prominent target of research because it is the primary component of one of the neuropathological hallmarks of Alzheimer's disease, neurofibrillary tangles. In vitro, tau was identified as a substrate of GSK3β in 1992 (Hanger et al., 1992; Mandelkow et al., 1992). The next year, a protein kinase that phosphorylated tau was isolated and was initially called tau protein kinase I, but this kinase was found to be GSK3β (Ishiguro et al., 1993), although the former nomenclature is still sometimes used in the literature. Subsequently, it was shown that tau was phosphorylated in intact cells by transfected GSK3β (Lovestone et al., 1994). Many additional studies have now found that GSK3β can phosphorylate as many as ten sites on tau (Hanger et al., 1998; reviewed in Johnson and Hartigan, 1998). Since lithium was found to be a selective inhibitor of GSK3β, there has been much interest in identifying changes in tau phosphorylation caused by lithium-induced inhibition of GSK3β as first shown by Klein and Melton (1996). For example, in cultured human NT2N neurons, treatment with lithium inhibited tau phosphorylation likely due to inhibition of GSK3β (Hong et al., 1997). Numerous

additional studies have found that lithium reduces the phosphorylation of tau (e.g., Munoz-Montano et al., 1997; Xie et al., 1998; Lovestone et al., 1999). Thus, many lines of evidence support the conclusion that tau is an in vivo substrate of GSK3β. This action of GSK3β can regulate the binding of tau to microtubules and thereby affect neuronal structure and neuronal plasticity, as well as potentially contribute to the neuropathology of Alzheimer's disease.

In addition to tau, both MAP1B and MAP2 are phosphorylated by GSK3β. Inhibition of GSK3β by lithium caused dramatic decreases in MAP1B phosphorylation (Lucas et al., 1998; Garcia-Perez et al., 1998). Furthermore, purified GSK3β was shown to directly phosphorylate MAP1B in vitro (Lucas et al., 1998). This phosphorylation of MAP1B was associated with significant changes in the localization of MAP1B, microtubule dynamics, and neuronal cytoarchitecture (Lucas et al., 1998; Garcia-Perez et al., 1998; Goold et al., 1999). In vitro, GSK3β phosphorylates MAP2 at serine-136 (Berling et al., 1994), threonine-1620, and threonine-1623 (Sanchez et al., 1996). Functionally, the GSK3β-mediated phosphorylation of MAP2 decreased the microtubule-binding and bundling capacity of MAP2 (Sanchez et al., 2000). Although less studied than tau, considering the importance of MAP1B and MAP2 in neuronal structure and plasticity (Johnson and Jope, 1992; Sanchez et al., 2000), it is likely that further important effects of GSK3β-mediated phosphorylation of MAPs on neuronal function will be identified.

Several other structural proteins have been reported to be phosphorylated by, or interact with, GSK3β. These include myelin basic protein (Yu and Yang, 1994), the neural cell-adhesion molecule (Mackie et al., 1989), neurofilament proteins (Guan et al.,

1991; Guidato et al., 1996), human dynamin-like protein (Hong et al., 1998; Chen et al., 2000a) and human Ninein, a centrosome-associated protein (Hong et al., 2000). However, the functional consequences of these interactions remain to be determined.

Overall, there is now abundant evidence that GSK3β exerts a substantial influence on neuronal structure, especially influencing dynamic changes in the cytoskeleton. It is intriguing to consider whether the therapeutic effects of lithium in bipolar disorder, an action likely involving neuronal plasticity, may be derived in part from lithium's influence on cytoskeletal dynamics stemming from its inhibitory effect on GSK3β, as recently reviewed (Jope, 1999a). Additionally, there is considerable evidence that GSK3β's actions on cytoskeletal proteins, notably tau, may contribute to the neuropathology of Alzheimer's disease. These potential associations with psychiatric and neurological diseases add further intrigue to the mysteriously diverse influences of GSK3β on neuronal function.

*Transcription factors and gene expression*

Perhaps one of the most important roles of GSK3β, as well as possibly the most surprising considering its inauspicious beginning, is that of a key regulator of a broad array of transcription factors, thereby extending its regulatory influence to the control of the expression of numerous genes. An impressively large number of transcription factors have been found to be regulated by GSK3β directly through phosphorylation of transcription factors themselves, and more are undoubtedly regulated indirectly as a consequence of changes in the activity of GSK3β. Transcription factors are considered critical targets of GSK3β because they have the critical role of transferring information

received by cells from the extracellular environment, which is often transduced by receptors and intracellular signaling pathways, to the nucleus to regulate a multitude of functions ranging from proliferation to apoptosis. The regulation of transcription factor activities plays several critical roles in relaying signals to the nucleus, functioning as (i) a filter to limit the magnitude of signals reaching the nucleus, (ii) an amplifier to increase the sensitivity of detection and/or response to particularly critical signals, (iii) an integrator, preventing a chaotic conglomeration of multiple overlapping and conflicting signals to convey a cohesive message, and (iv) an interpreter to translate numerous types of signals to a form that the nucleus can comprehend. These critical functions are fulfilled in part by transcription factors, proteins that, when activated, bind specific DNA sequences to regulate transcription. Thus, the regulatory effects of GSK3β on transcription factors can have a tremendous impact on cell function.

*Activator protein-1 (AP-1).* AP-1 refers to a family of basic leucine zipper transcription factors originally characterized as mediating phorbol ester tumor promoter-induced gene expression (Angel et al., 1987). These transcription factors are composed of homodimer or heterodimer protein complexes, primarily consisting of members of the Jun, Fos, or ATF families of proteins that are encoded by immediate early genes (Pennypacker et al., 1995; Karin et al., 1997). Since the initial discovery of these transcription factors, innumerable studies have emerged demonstrating that AP-1 activity is influenced by a wide variety of stimuli, including activation of many neurotransmitter and growth factor receptors, neuronal activity, toxic stimuli, and other signaling processes (Morgan et al., 1987; Dragunow et al., 1990). AP-1 regulates the expression of

a diverse array of genes including ones involved in cell growth, proliferation, and in some instances, cell death (Karin et al., 1997).

Because of its importance to a wide array of cellular functions, AP-1 activity is strictly regulated at both the transcriptional and posttranscriptional levels. The primary posttranscriptional mechanism of AP-1 regulation is phosphorylation. Phosphorylation of Jun at serine-73 and serine-63 located within the N-terminal transactivation domain increases its ability to activate transcription (Smeal et al., 1991; Deng and Karin, 1994). Identified kinases thought to phosphorylate these sites and increase AP-1 activity include protein kinase C, extracellular-related kinases (ERK 1 and 2), and activated Ras, among others (Boyle et al., 1991; Pulverer et al., 1991; Deng and Karin, 1994). In contrast to these kinase-dependent activating modifications, GSK3β has been implicated in the phosphorylation and negative regulation of AP-1 activation. Boyle et al. (1991) reported that GSK3β phosphorylated c-Jun in vitro at three sites, including threonine-239, serine-243, and serine-249, and decreased its DNA binding activity. This study further showed that mutation of serine-243 to phenylalanine blocked phosphorylation of all three sites by GSK3β and restored c-Jun activity (Boyle et al., 1991). Additional studies have reported that expression of GSK3β in cultured cells significantly attenuated the DNA-binding and transcriptional activation functions of c-Jun and, to a lesser extent, other Jun-related proteins including JunB, JunD, and v-Jun, resulting in decreased AP-1 activity (de Groot et al., 1993; Nikolakaki et al., 1993). Evidence suggests that other cellular kinases can counteract the inhibition of Jun by GSK3β to precisely regulate AP-1 activity. Activation of protein kinase C decreased phosphorylation of sites negatively regulated by GSK3β, resulting in increased AP-1 activity in vitro (Boyle et al., 1991). Furthermore, upon

exposure of HEK-293 cells to fibronectin, integrin-linked kinase directly phosphorylated and inhibited GSK3β activity, resulting in increased AP-1 activity (Troussard et al., 1999). Taken together, findings from these studies implicate GSK3β as a primary kinase involved in the negative regulation of AP-1 activity and also point to the coordinated action of GSK3β and other kinases as mechanisms of regulation of AP-1, a transcription factor critical to many cellular processes.

Several studies have examined the effects of lithium on the activation of AP-1, an action that may be due to lithium's inhibition of GSK3β. This effect of lithium would be predicted to attenuate GSK3β-induced inhibition of AP-1, thus leading to enhanced AP-1 activity after lithium treatment. In accordance with this, there have been several reports of increased AP-1 activity in cultured cells and in rat brain after treatment with lithium (Jope and Song, 1997; Ozaki and Chuang 1997; Hedgepeth et al., 1997; Asghari et al., 1998; Yuan et al., 1998). For example, Hedgepeth et al. (1997) found that lithium treatment increased AP-1-driven transcription, likely due to blockade of the inhibitory phosphorylation by GSK3β of the Jun protein component of AP-1. In accordance with these studies, the GSK3β inhibitor lithium concentration-dependently increased AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells (Fig. 6A). However, although the increased AP-1 activity shown here and found in several reports is consistent with lithium causing a blockade of GSK3β's inhibition of AP-1, these studies are complicated by the fact that lithium also has other sites of action that may affect AP-1 DNA binding activity (Jope, 1999a), and none of these studies have directly demonstrated that inhibition of GSK3β accounts for the observed actions of lithium.

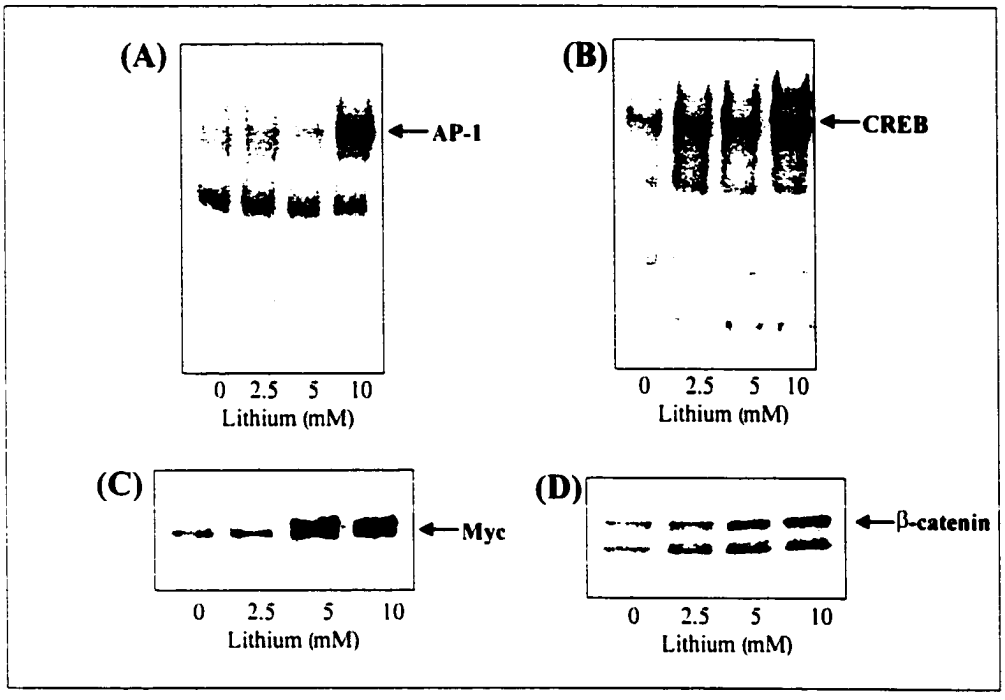


**FIG. 6.** Effects of lithium on transcription factors. Nuclear fractions from serum-starved human neuroblastoma SH-SY5Y cells were treated with 0, 2.5, 5, or 10 m*M* lithium for 1 h and assessed for **A:** AP-1 DNA binding activity, **B:** CREB DNA binding activity, **C:** Myc protein levels, and **D:** β-catenin protein levels. DNA binding was measured using the electrophoretic mobility shift assay as described previously (Bijur et al., 2000), and Myc and β-catenin protein levels were measured by immunoblot analyses. These results demonstrate that lithium treatment up-regulates each of these transcription factors. In addition, the data also demonstrate that there are differences among GSK3β-regulated transcription factors in sensitivity to lithium, with a sensitivity ranking of CREB > β-catenin > Myc > AP-1.

The modulation of AP-1 activity by lithium has raised speculation that this might be associated with lithium's therapeutic effect in bipolar disorder.

*Cyclic AMP response element binding protein (CREB).* The nuclear phosphoprotein CREB was the first identified member of the basic leucine zipper family of transcription factors that modulate the expression of genes containing promoters with cyclic AMP response elements (Montminy and Bilezikjian, 1987; Waeber and Habener, 1991; Brindle and Montminy, 1992). CREB contains a C-terminal basic region and leucine zipper that promote DNA recognition and binding to the CRE motif 5'-TGACGTCA-3', and an N-terminal transcriptional activation domain containing multiple phosphorylation sites for a variety of kinases (Gonzalez et al., 1989; Yun et al., 1990). Phosphorylation within this kinase-inducible domain of CREB triggers recruitment of the coactivator CREB-binding protein (CBP) and culminates in the transcription of CREB-regulated genes (Chrivia et al., 1993; Arias et al., 1994; Ferreri et al., 1994; Kwok et al., 1994; Parker et al., 1996).

CREB regulates many critical processes, such as formation of long-term memory, maintenance of synaptic plasticity, and apoptosis (Struthers et al., 1991; Davis et al., 1996; Deisseroth et al., 1996; Silva et al., 1998; Bevilaqua et al., 1999; Shaywitz and Greenberg, 1999). Several groups have demonstrated that long-term memory formation is blocked by the introduction of inactive mutated CREB isoforms or CREB antisense oligonucleotides (Kaang et al., 1993; Bourtchuladze et al., 1994; Yin et al., 1994; Blendy et al., 1996; Guzowski and McGaugh, 1997). CREB-null mice expressing functionally inactive CREB die immediately after birth (Rudolph et al., 1998). Antiapoptotic actions

of CREB include the findings that human melanoma cells expressing a dominant-negative CREB mutant had increased susceptibility to thapsigargin-induced apoptosis (Jean et al., 1998), that inhibition of CREB activation promotes apoptosis (Bonni et al., 1999). and that CREB blocked okadaic acid-induced apoptosis in PC12 cells (Walton et al., 1999). Additionally, multiple reports suggest that CREB promotes cell survival by up-regulating expression of the antiapoptotic protein bcl-2 (Ji et al., 1996; Wilson et al., 1996; Pugazhenthi et al., 1999; Riccio et al., 1999; Pugazhenthi et al, 2000). It is evident from these many important actions of CREB that regulation of its activity as a transcription factor is critical for numerous cellular functions.

CREB activity is regulated by complex phosphorylation mechanisms that are not completely characterized (Shaywitz and Greenberg, 1999). Gonzalez and Montminy (1989) discovered that phosphorylation of CREB at serine-133, located within its N-terminal transcriptional activation domain, is required for CREB to be transcriptionally active. In accordance with this finding, several groups have identified numerous signaling systems that activate CREB through phosphorylation of serine-133 (Sheng et al., 1991; Alberts et al., 1994; Ginty et al., 1994; Xing et al., 1996). Wang et al. (1994c) further demonstrated that phosphorylation of CREB at serine-133 created a consensus site for phosphorylation by GSK3β at serine-129, providing an example of the hierarchical phosphorylation often observed with GSK3β. Only two studies have addressed the functional consequences of this secondary phosphorylation of CREB by GSK3β. Fiol et al. (1994) provided evidence that GSK3β facilitated activation of CREB. They demonstrated that transcriptional activation by CREB in response to cyclic AMP was potentiated in F9 cells overexpressing wild-type GSK-3β and was impaired in PC12

cells transfected with CREB containing a mutation in the GSK3β phosphorylation site (Fiol et al., 1994). In contrast, Bullock and Habener (1998) found that phosphorylation of CREB by GSK3β attenuated protein kinase A-induced CREB DNA binding activity. Thus, although there is a consensus that CREB is phosphorylated by GSK3β, the functional outcome of this modification remains to be clarified. Additionally, it was recently discovered that protein kinase A inhibits GSK3β by direct phosphorylation of serine-9 (Fang et al., 2000), indicating that activation of protein kinase A both stimulates CREB activation and inhibits the activity of GSK3β.

Studies of the effects of lithium on CREB DNA binding activity may contribute to clarifying the contradictory findings concerning the influence of GSK3β on CREB activation discussed above, but there are only a few such studies. Ozaki and Chuang (1997) reported results consistent with an inhibitory effect of GSK3β on CREB activity that can be reversed by lithium treatment. They found that lithium treatment increased CREB DNA binding activity in cultured cerebellar granule cells and in several regions of rat brain (Ozaki and Chuang, 1997). In contrast, Wang et al. (1999) reported that lithium had no effect on basal, but attenuated forskolin-stimulated, CREB DNA binding activity in human neuroblastoma SH-SY5Y cells. These apparently contradictory findings may be due to the facts that multiple signaling pathways can regulate CREB DNA binding activity, including factors in serum present in the study by Wang et al. (1999), and that lithium can modulate more than just the GSK3β regulatory component (Jope, 1999a). Recent evidence (Grimes and Jope, 2000) has revealed that when CREB DNA binding activity in SH-SY5Y cells is measured after lithium treatment, enhanced CREB DNA binding activity caused by lithium is evident only after withdrawal of serum, which

removes the signaling pathways activated by growth factors and hormones present in serum. Fig. 6B shows that treatment with lithium concentration-dependently increases CREB DNA binding activity and, further, that CREB activation is much more sensitive to lithium than is AP-1 activation. Taken together, these studies indicate that CREB DNA binding activity is regulated by a complex mixture of signals, one of which encompasses an inhibitory influence of GSK3β that can be mitigated by lithium.

*Heat shock factor-1 (HSF-1).* The transcription factor HSF-1 is one of the most crucial signaling components mediating cellular defense mechanisms in response to potential lethal stressors. HSF-1 is activated in response to many stressors, including oxidative stress and heavy metals, in addition to the commonly employed experimental paradigm of exposing tissues to elevated temperatures. Activation of HSF-1 in response to stress is important because it controls the expression of heat shock proteins, such as hsp70, which chaperone misfolded proteins to avoid their accumulation within cells. The stimulation and inhibition of HSF-1 activity is controlled by complex phosphorylation and dephosphorylation modifications that are not fully elucidated. Nonetheless, there is an increasing amount of evidence that GSK3β has an inhibitory effect on the activation of HSF-1. Thus, stress-induced activation of Akt and the ensuing serine-9 phosphorylation and inhibition of GSK3β appear to be important components of the HSF-1-activating signal caused by a variety of types of cellular stress.

The involvement of GSK3β in regulating HSF-1 activity was first detected by Chu et al. (1996). Following the discovery that ERK activity is inhibitory for HSF-1 activation (Mivechi and Giaccia, 1995), Chu et al. (1996, 1998) discovered that

inactivation of HSF-1 could result from hierarchical phosphorylation, with ERK-induced phosphorylation of serine-307 of HSF-1 being required for the subsequent GSK3β-mediated phosphorylation of serine-303 of HSF-1. They suggested that activation of HSF-1 would be associated with dephosphorylation of serine-303 and that serine-303 could be rephosphorylated during the recovery stage after stress, when heat shock transcription becomes deactivated. However, Xia et al. (1998) suggested that phosphorylation on serine-303 could not occur when HSF-1 was in the activated state but may serve primarily to maintain HSF-1 in the basal, inactive conformation. Kline and Morimoto (1997) also found that constitutive phosphorylation of serine residues 303 and 307 of HSF-1 repressed activity. He et al. (1998) suggested that GSK3β contributed to deactivation of HSF-1 because transient overexpression of GSK3β in HeLa cells increased the rate of inactivation of HSF-1 after heat shock. They further showed that ERK activity was necessary for the inactivating effect of GSK3β, further supporting the concept that ERK phosphorylation of HSF-1 precedes phosphorylation by GSK3β. He et al. (1998) also reported that GSK3β activity increased dramatically during the recovery phase after heat shock, thus providing a mechanism for inactivation of HSF-1 following heat stress. In the first examination of the influence of GSK3β on HSF-1 activation in a neuronal model system, Bijur and Jope (2000) found that overexpression of GSK3β severely impaired activation of HSF-1 and the subsequent expression of hsp70. This study further provided the first evidence that lithium influences the activation of neuronal HSF-1 and heat shock protein expression by reducing the inhibitory influence of GSK3β on these responses to stress (Bijur and Jope, 2000). Similar results were recently obtained using *Xenopus* oocytes (Xavier et al., 2000). This very interesting report also showed that

overexpression of GSK3β impaired activation of HSF-1, whereas lithium was facilitory, and suggested that GSK3β is a terminal kinase for several stress signal-transduction pathways that regulate HSF-1 activity (Xavier et al., 2000). Thus, although precise mechanisms remain to be established, these findings have solidified the concept that GSK3β is an important inhibitory regulator of HSF-1.

Little is known about how this inhibitory regulation by GSK3β through phosphorylation of HSF-1 interacts with the complex stimulatory phosphorylation modifications of HSF-1, which can be induced by a variety of protein kinases, such as p38 (Hung et al., 1998) and protein kinase C (Ding et al., 1996, 1997; Holmberg et al., 1997; Xia and Voellmy, 1997; but see Chu et al., 1998 for an opposing point of view). For example, protein kinase C phosphorylates and inhibits GSK3β, an action that theoretically may contribute to the activating effect of protein kinase C on HSF-1 in addition to a direct protein kinase C-mediated phosphorylation of HSF-1. Thus, although discrepancies in the literature suggest that further clarification of the mechanisms by which phosphorylation regulates HSF-1 activity, substantial evidence indicates that GSK3β contributes to inhibitory control of HSF-1's function as a transcription factor. Furthermore, initial findings suggest that a part of the neuroprotective effect of lithium may be contributed by lithium's attenuation of GSK3β-induced inhibition of HSF-1 activation and heat shock protein expression (Bijur et al., 2000; Bijur and Jope, 2000).

*Nuclear factor of activated T cells (NFAT).* NFAT is a family of rapidly inducible transcription factors including four structurally related, predominantly cytosolic members, NFAT1/NFATp, NFAT2/NFATc, NFAT3, and the predominantly nuclear,

constitutively active NFAT5. Although initially characterized in Jurkat T cells, NFAT proteins are expressed in a variety of cells and tissues both within and outside the immune system (reviewed in Rao et al., 1997).

Structural features of NFAT proteins include a DNA binding domain homologous to that of Rel-family proteins that facilitates monomeric binding to the DNA consensus sequence GGAAA, and an acidic, proline-rich regulatory domain that mediates NFAT transcriptional activation (Shaw et al., 1988; Hoey et al., 1995; Jain et al., 1995; Luo et al., 1996a). NFAT activity is directly regulated by the serine/threonine calcium-activated phosphatase calcineurin (Mattila et al., 1990; Jain et al., 1993; McCaffrey et al., 1993). In resting cells, NFAT is phosphorylated, localized in the cytosol, and has low affinity for DNA binding. In response to calcium mobilization, activated calcineurin directly binds and dephosphorylates NFAT, resulting in nuclear translocation, stimulation of NFAT DNA binding activity, and increased transcription of NFAT-regulated genes (Ruff and Leach, 1995; Loh et al., 1996; Luo et al., 1996b; Shibasaki et al., 1996; Wesselborg et al., 1996).

Phosphorylation of NFAT is required for nuclear export of the transcription factor when calcium-calcineurin signaling is terminated, suggesting the involvement of an unknown kinase in the deactivation of NFAT. Two studies examining the identity of this kinase implicated GSK3β in the phosphorylation, deactivation, and subsequent nuclear export of NFAT (Beals et al., 1997; Graef et al., 1999). Beals et al. (1997) discovered that GSK3β could stoichiometrically phosphorylate NFATc in purified brain extracts, but only after NFATc was phosphorylated by a priming kinase, providing an example of hierarchical phosphorylation by GSK3β. This study also showed that overexpression of

GSK3β in COS cells blocked the calcineurin-induced nuclear translocation of NFATc in response to calcium ionophore treatment (Beals et al., 1997). Graef et al. (1999) examined mechanisms of NFATc export in hippocampal neurons and found that overexpression of GSK3β inhibited NFAT-dependent transcription in response to phorbol ester and calcium ionophore treatments. The authors determined that this inhibition was specific to GSK3β activity since cotransfection of neurons with several other kinases did not inhibit stimulation of NFATc transcription (Graef et al., 1999). This study further demonstrated that NFATc activation was increased in hippocampal neurons overexpressing a dominant-negative mutant of GSK3β (Graef et al., 1999). Results from both studies provide strong evidence that GSK3β phosphorylates, deactivates, and induces nuclear export of NFATc, providing a mechanism by which calcium-calcineurin signaling and GSK3β could counteract each other to regulate NFATc activity. Regulation of NFAT by GSK3β was linked to hypertrophy in myocytes (Haq et al., 2000). However, little is known concerning the specificity of GSK3β for different NFAT isoforms. del Arco et al. (2000) demonstrated that the p38 MAP kinase phosphorylates, deactivates, and induces nuclear export of NFATp, but not NFATc, in intact lymphocytes, suggesting that phosphorylation and deactivation of specific NFAT isoforms might be regulated by different kinases. NFAT induces IL-2 production and lithium, an inhibitor of GSK3β, enhances T cell production of IL-2 and proliferation (Kucharz et al., 1988; Wu and Yang, 1991; Ohteki et al., 2000), likely by blocking the inhibitory effect of GSK3β on NFAT activity (Yost et al., 1998). These findings indicate that lithium's effects on certain cytokines in the immune system, as well as in the central

nervous system, may derive from its inhibition of GSK3β and the consequential elevated activation of NFAT.

*Myc.* The Myc transcription factor is a short-lived, nuclear phosphoprotein product of the myc gene family, which includes c-myc, N-myc, L-myc, the distantly related s-myc, and the nonfunctional B-myc that regulates cellular growth and differentiation and, when disregulated, contributes to cellular transformation (Meichle et al., 1992; Dang et al., 1999). Important structural features of Myc include a basic region, a helix-loop-helix domain, and a leucine zipper motif, all located within the C-terminal region, which facilitate dimerization and DNA binding. There is also an N-terminal transactivation domain, a proline-rich region highly conserved among the Myc family of proteins, responsible for regulation of Myc transcriptional activation (Meichle et al., 1992; Henriksson et al., 1993; Dang et al., 1999). Myc binds specifically to the DNA consensus sequence CACGTG in concert with the smaller and more stable nuclear phosphoprotein Max. Max complexes with c-Myc, L-Myc, and N-Myc, but not with other proteins of similar structure, and Myc/Max heterodimers bind DNA more efficiently than Max/Max homodimers (Blackwell et al., 1990; Blackwood and Eisenman, 1991; Halazonetis and Kandil, 1991; Wenzel et al., 1991; Papoulas et al., 1992).

Phosphorylation is thought to regulate Myc transcriptional activation. Mutagenesis analyses demonstrated that Myc is regulated by phosphorylation of serine and threonine residues located within its N-terminal transcriptional activation domain. Kinases implicated in phosphorylation of Myc within this domain include c-Jun N-

terminal kinase (JNK), ERKs, cell cycle-dependent protein kinase-2, and GSK3β (Seth et al., 1991; Saksela et al., 1992; Henriksson et al., 1993; Pulverer et al., 1994; Noguchi et al., 1999). In vitro studies demonstrated that GSK3β can phosphorylate serine residues 38, 42, and 62, and threonine residue 58 within the transactivation domain of Myc, sites considered important for regulation of Myc transcriptional activity in vivo (Saksela et al., 1992; Henriksson et al., 1993; Pulverer et al., 1994). Phosphorylation of threonine-58 and serine-62 by GSK3β was reported in two independent studies to negatively regulate Myc function, resulting in decreased cellular growth and transformation (Henriksson et al., 1993; Pulverer et al., 1994). However, in an earlier study, phosphorylation of Myc at serine-62 in response to growth factor stimulation was reported to increase transactivation of gene expression by Myc, although the kinases implicated in this response were the ERKs and cell cycle-dependent kinase-2 rather than GSK3β (Seth et al., 1991). Thus, it is evident that Myc is likely an in vivo substrate for GSK3β, but the sites phosphorylated in vivo and the functional outcome remain to be more definitively established.

The importance of the transactivation domain in regulating Myc function and the evidence that GSK-3β can phosphorylate multiple sites within this domain provide preliminary evidence that GSK3β may be an important mediator of Myc function and regulation. To investigate this interaction further, our laboratory has investigated whether in intact cells, changes in GSK3β activity modulated the level of Myc in the nucleus. In human neuroblastoma SH-SY5Y cells, inhibition of GSK3β by lithium caused increases of Myc nuclear protein levels (Fig. 6C). These results corroborate the earlier evidence that GSK3β has an inhibitory influence on Myc function and reveal that lithium, a

primary therapeutic drug for the treatment of bipolar mood disorder, facilitates Myc function.

*β-catenin.* β-Catenin, a member of the catenin family of proteins that also includes α- and γ-catenin, for many years was considered exclusively as a cell adhesion molecule because of its original characterization as a plasma membrane protein that anchors cadherins during the formation of cell adherens junctions (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). However, more recent research has made it apparent that β-catenin has an equally significant role in cell signaling, completely independent from its capacity as an adhesion molecule, which has been most completely defined in the Wnt signaling pathway. Evidence from several studies indicates that Wnt activation results in the stabilization and accumulation of β-catenin, thereby increasing the availability of β-catenin to bind proteins of the Tcf-Lef transcription factor family (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). The binding of β-catenin to Tcf-Lef proteins initiates translocation of the complex to the nucleus, where it binds DNA and transcriptionally regulates Tcf-Lef target genes.

Like many transcription factors, β-catenin levels are subject to regulation by phosphorylation. A substantial amount of evidence points to GSK3β as a primary kinase responsible for phosphorylation and down-regulation of β-catenin levels (Yost et al, 1996; Rubinfeld et al., 1996; Sakanaka et al., 1998). For example, in *Xenopus*, phosphorylation of β-catenin requires a GSK3β site, β-catenin mutants lacking this site are more active and stable than wild-type β-catenin in the presence of GSK3β activity, and inhibition of endogenous GSK3β with a dominant negative mutant results in

increased β-catenin levels (Yost et al., 1996). Additional studies in SW 480 colon cancer cells showed that in response to high levels of accumulated β-catenin, GSK3β, in concert with other regulators including Axin and APC, could bind and phosphorylate β-catenin, resulting in destabilization and degradation of β-catenin protein levels and, consequently, inactivation of β-catenin/Tcf-Lef-induced transcription (Rubinfeld et al., 1996; Sakanaka et al., 1998). β-Catenin associated with Tcf-Lef transcription factors regulates the expression of a variety of genes including those encoding proteins involved in such processes as cell cycle regulation, cell adhesion, and cellular development (reviewed in Novak and Dedhar, 1999). Additionally, irregular β-catenin/Tcf-Lef-induced transcription, as a result of mutations in β-catenin, has been implicated in tumor formation, specifically in colorectal cancers (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al, 1997). It is evident from these studies that β-catenin has numerous, critical cellular functions, and thus must be subject to precise regulatory mechanisms, one of which includes negative control by GSK3β through phosphorylation and subsequent destabilization of β-catenin. Thus, inhibition of GSK3β with lithium leads to increased β-catenin, as exemplified by the results shown in Fig. 6D.

*CCCAAT/enhancer binding protein (C/EBP).* The six identified isoforms of C/EBP, including α, β, γ, δ, ε, and ζ, make up a family of transcription factors that contribute to the regulation of cellular differentiation in a variety of tissues. C/EBP proteins are composed of C-terminal leucine zippers that facilitate dimerization with other C/EBP proteins or members of the NFκB or Fos/Jun transcription factor families, and an N-terminal region that regulates C/EBP activation and function (reviewed in

Lekstrom-Himes and Xanthopoulos, 1998). C/EBP transcription factors are expressed in a variety of tissues and are most well known for their regulation of genes involved in nutrient metabolism in the liver and cellular differentiation in adipocytes. C/EBP expression has been detected in the adult brain, specifically in the hippocampus and cortex (Kuo et al., 1990; Yukawa et al., 1998). However, few studies have addressed the role of C/EBP proteins in neuronal signaling. Walton et al. (1998) demonstrated that C/EBPα DNA binding activity is increased in microglial cells, but not astrocytes or neurons, following ischemic brain injury. Additionally, Yukawa et al. (1998) showed that stimulation of signals that potentiate long-term memory formation, such as cyclic AMP or calcium, increased both C/EBP-β and -δ expression and DNA binding activities in rat hippocampal neurons. These findings provide evidence that C/EBP transcription factors may regulate the expression of genes important to neuronal signaling.

Similar to many transcription factors, C/EBP activity is regulated by phosphorylation. However, little is known concerning the kinases that regulate C/EBP. Yukawa et al. (1998) showed that calcium/calmodulin-dependent kinase IV stimulates C/EBP-β and -δ expression and DNA binding activities in rat hippocampal neurons. Additionally, multiple studies have demonstrated rapid dephosphorylation of C/EBPα, with subsequent decreases in C/EBPα DNA binding activity and expression, in response to insulin (MacDougald et al., 1995; Hemati et al., 1997). Ross et al. (1999) identified GSK3β as a primary kinase involved in insulin regulation of C/EBP activity. They demonstrated that insulin, through activation of PI3K and protein phosphatase 1, inactivated GSK3β, resulting in dephosphorylation of the two sites of C/EBPα phosphorylated by GSK3β, threonine-222 and threonine-226 (Ross et al., 1999). They

also showed that GSK3β is required for C/EBPα-induced adipocyte differentiation since inhibition of GSK3β with lithium blocked differentiation of mouse 3T3-L1 preadipocytes (Ross et al., 1999). These results indicate that C/EBPα is regulated by GSK3β substantially differently from other transcription factors, including the evidence of threonine-phosphorylation of a transcription factor by GSK3β that is associated with activation, not inhibition, of transcription factor function. However, these findings provide only limited insight concerning the regulation of C/EBP proteins by GSK3β, and future investigations could afford further information concerning C/EBP transcription factors and their regulation by GSK3β in the context of neuronal signaling.

*NFκB.* The NFκB/Rel transcription factors include NFκB1 (p50), NFκB2 (p52), Rel A (p65), rel B, and c-rel. These proteins share a common structural Rel homology domain that regulates dimerization, interactions with IκB inhibitory proteins, and DNA binding (reviewed in Baldwin, 1996; Grilli and Memo, 1999). NFκB transcription factors bind DNA as homodimers or heterodimers, though the most frequently occurring and most thoroughly studied NFκB dimer consists of NFκB1 (p50) and Rel A (p65) (Baeuerle and Baltimore, 1989; Urban et al., 1991). Early investigations of NFκB activity, primarily conducted in immune cells since NFκB was originally identified in mature B lymphocytes (Sen and Baltimore, 1986), found that NFκB transcription factors regulate genes involved in immune and inflammatory responses (reviewed in Baeuerle and Henkel, 1994). However, numerous additional studies have shown that NFκB-regulated gene expression is a critical component of neuronal function (reviewed in Lezoualc'h and Behl, 1998).

NFκB activity is precisely regulated by its interaction with the inhibitory IκB proteins (Baeuerle and Baltimore, 1988; reviewed in Baldwin, 1996). Under basal conditions, NFκB is localized in the cytoplasm bound to the ankyrin repeat regions of IκB that conceal the nuclear localization signal of NFκB, preventing its import by the nucleus (Beg et al., 1992; Ganchi et al., 1992). Stimuli that activate NFκB do so by increasing the phosphorylation and ubiquitin-dependent proteolysis of IκB, releasing NFκB for nuclear translocation and subsequent transcription of NFκB-regulated genes (Henkel et al., 1993; Beg et al., 1993; Palombella et al., 1994). Several IκB kinases have been identified that directly phosphorylate IκB proteins on critical serine residues, resulting in degradation of IκB and activation of NFκB (DiDonato et al., 1997; Mercurio et al., 1997; Regnier, et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). However, much remains to be learned about regulatory mechanisms upstream and downstream of IκB kinase activity.

Two recent reports revealed that NFκB activity is subject to regulation by GSK3β. Hoeflich et al. (2000) found that GSK3β-null mice demonstrated increased sensitivity to tumor necrosis factor α (TNFα)-induced toxicity and that TNFα-induced NFκB DNA binding activity, which promotes cell survival, was significantly decreased in GSK3β-null embryonic fibroblasts compared with wild-type cells. Additionally, lithium-induced inhibition of GSK3β resulted in decreased TNFα-induced NFκB activity in HEK293 cells. The decrease in NFκB activity in GSK3β null cells occurred downstream of IκB phosphorylation and translocation of NFκB to the nucleus (Hoeflich et al., 2000). These findings indicate that GSK3β activity is necessary for TNFα-induced

NFκB activation. However, another recent study demonstrated an inhibitory influence of GSK3β on NFκB. Bournat et al. (2000) examined the role of GSK3β in Wnt-1-induced NFκB activity in PC12 cells. They demonstrated that Wnt-1 expression increased PC12 cell survival in an NFκB-dependent manner and that inhibition of GSK3β with lithium or the expression of dominant-negative GSK3β mimicked Wnt-1 signaling, resulting in increased NFκB activity and PC12 cell survival (Bournat et al., 2000). Thus, in contrast to the findings by Hoeflich et al. (2000), these findings indicate that GSK3β negatively regulates NFκB activity (Bournat et al., 2000). Taken together, these reports appear to reach opposite conclusions concerning the regulatory role of GSK3β on the activation of NFκB. The differences may indicate a dependence of GSK3β's modulatory effect on the signaling systems that lead to the activation of NFκB, but further studies will be required to clarify the regulatory role of GSK3β on the activity of NFκB.

*Summary.* Overall, there is substantial evidence that GSK3β regulates the activity of a remarkable array of transcription factors (Fig. 7). However, there are distinct differences among transcription factors in sensitivity to GSK3β and its inhibition by lithium. This point has received little experimental attention, but, as exemplified in Fig. 6, these are large differences which likely affect the signaling systems most vulnerable to alterations in GSK3β activity and most amenable to modulation by lithium. Interestingly, in most cases GSK3β is inhibitory toward transcription factor activation, and many of the transcription factors subject to inhibitory regulation by GSK3β are key components of neuronal survival mechanisms. It is apparent that modulation of transcription factor

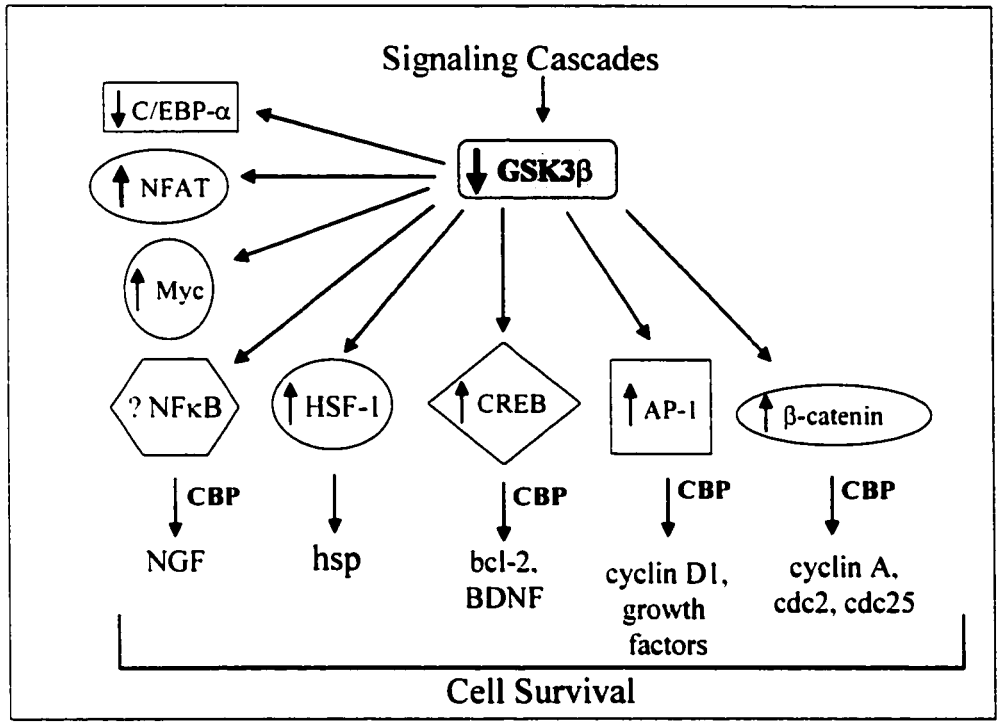


**FIG. 7.** Transcription factors regulated by GSK3β. The effects of inhibition of GSK3β are shown on transcription factors, including several that are known to promote cell survival.

activity is likely an important mechanism by which GSK3β, and modulators of GSK3β, affects neuronal plasticity and survival.

## OUTCOMES OF GSK3β MODULATION

### *Mood Disorders*

The major impetus for the concept that GSK3β may be involved in psychiatric disorders, and especially in bipolar mood disorder, derives from the finding that lithium, the primary therapeutic agent for bipolar mood disorder, is a selective inhibitor of GSK3β (Klein and Melton, 1996; Stambolic et al., 1996). The possibility that GSK3β is a relevant target for mood stabilizers was further substantiated by the finding that valproate, which has recently been shown to be an effective therapy for bipolar disorder, also inhibits GSK3β (Chen et al., 1999a). It is noteworthy that the inhibition of GSK3β by lithium, as well as by valproate, occurs at concentrations that are within the therapeutic range, with approximately 2 m*M* lithium causing a 50% inhibition of GSK3β (Klein and Melton, 1996). This suggests that therapeutic levels of lithium, near 1 mM, would effectively dampen, but not completely inhibit, GSK3β-mediated signaling events. This seems to be a logical manner for a mood stabilizer to act, lessening the magnitude of signals without entirely blocking critical signaling mechanisms, as recently proposed (Jope, 1999b). This raises several possibilities: that GSK3β may be a critical target for the therapeutic actions of lithium and other mood stabilizing drugs, that GSK3β levels or activity may be altered in certain psychiatric disorders, that dysfunctions in signaling systems that regulate the activity of GSK3β could contribute to mood disorders, and that there may be abnormal activities of effectors downstream of GSK3β that are amenable to

correction by lithium's inhibitory effects on GSK3β. These possibilities are currently the focus of many investigations, and concrete conclusions have yet to be drawn.

Identifying which, if any, of these possible links between GSK3β and bipolar disorder, or the therapeutic effects of lithium, occur is a difficult but compelling issue. GSK3β levels in studies of psychiatric disorders can be assessed only by using samples of postmortem brain, an approach fraught with difficulties (as discussed in Pacheco and Jope, 1996). Nevertheless, two studies have used this method, reporting that GSK3β levels were unaltered in postmortem brains from subjects with bipolar disorder compared with control subjects (Lesort et al., 1999a; Kovlovsky et al., 2000). Interestingly, GSK3β levels were recently reported to be significantly lower in the postmortem frontal cortex from subjects with schizophrenia, but not bipolar disorder or unipolar depression, compared with control subjects (Kovlovsky et al., 2000). Further investigations of GSK3β in bipolar disorder and other psychiatric illnesses are clearly warranted considering the many mechanisms capable of regulating its activity and the many substrates of GSK3β.

As discussed in previous sections of this article, there are numerous substrates phosphorylated by GSK3β, providing an equally great number of targets for lithium to influence cellular metabolism, signaling, and gene expression through its inhibitory effect on GSK3β. There is substantial evidence that lithium alters GSK3β-mediated phosphorylation of microtubule associated proteins, such as tau. Phosphorylation of these proteins alters their association with microtubules, which in turn regulates cytoskeletal dynamics, thus influencing neuronal plasticity. It is intriguing to consider the possibility

that such regulation of neuronal plasticity may be an important component in therapeutic actions of lithium and valproate.

Equally intriguing are the possibilities engendered by the multiple transcription factors regulated by GSK3β and thus subject to modulation by lithium. This has contributed to much interest in potential regulatory effects of lithium on the activation of several transcription factors. Most of the studies of lithium's effects on transcription factors have used high concentrations of lithium that are well above therapeutic levels (i.e., near 1 m*M* lithium) as a means to detect the regulatory effects of GSK3β. However, the use of relatively high lithium concentrations to facilitate experimental detection of effects does not negate the potential impact of smaller changes elicited by lower concentrations of lithium that are within the therapeutic range. Additionally, several studies have used therapeutically relevant concentrations of lithium in an attempt to determine whether these are capable of modulating transcription factor activities, mostly in studies of AP-1. Activation of AP-1 has been found in rat brain and cultured cells after administration of a wide range of doses of lithium (Jope and Song, 1997; Ozaki and Chuang, 1997; Hedgepeth et al., 1997; Asghari et al., 1998; Yuan et al., 1998). Although the mechanism causing this effect was not specifically identified, this response to lithium is consistent with it being the result of inhibition of GSK3β, which would relieve the inhibitory effect of GSK3β on AP-1 activation. The CREB transcription factor also has been studied as a potential downstream target of lithium's actions, but with mixed results. Lithium enhanced CREB activation in cultured cerebellar granule cells and rat brain (Ozaki and Chuang, 1997), but not in SH-SY5Y neuroblastoma cells (Wang et al., 1999). The latter finding may be due to the use of cells maintained in serum-containing media

because growth factors in serum keep Akt active, which maintains GSK3β in an inactive state, thus preventing detection of the effects of lithium's inhibition of GSK3β. Active GSK3β strongly inhibits CREB activation, and this inhibition is overcome by lithium, which leads to facilitation of CREB activation. CREB is an especially interesting potential target for the therapeutic effects of lithium because other lines of research have provided evidence that CREB is a key component in mood disorders and their amelioration by mood stabilizers and antidepressants. For example, Duman et al. (1997) presented an eloquent synthesis of the potentially critical role of CREB in depression and its elevated activation induced by antidepressants.

In addition to AP-1 and CREB, the potential importance of other transcription factors that are regulated by GSK3β, and thus susceptible to modulation by lithium, should not be overlooked. Along with identifying those transcription factors influenced by therapeutic concentrations of lithium, a challenge for the future will be to integrate the changes in multiple transcription factors that can be influenced by lithium's inhibition of GSK3β, along with the cytoskeletal effects and alterations induced on other GSK3β substrates, into a comprehensive description of lithium's mood-stabilizing mechanism of action.

At first glance, this may seem to be a bewildering array of potential effects of lithium (Figure 8), and one may wonder which of these is the single key target. However, we have previously proposed that, considering the complexities of mood disorders and the multiple therapeutic actions of lithium, its therapeutically relevant action is likely not limited to alteration of a single signaling pathway but is more likely to be the culmination of multiple effects (Jope, 1999b). Thus, by adjusting the strength and balance of multiple

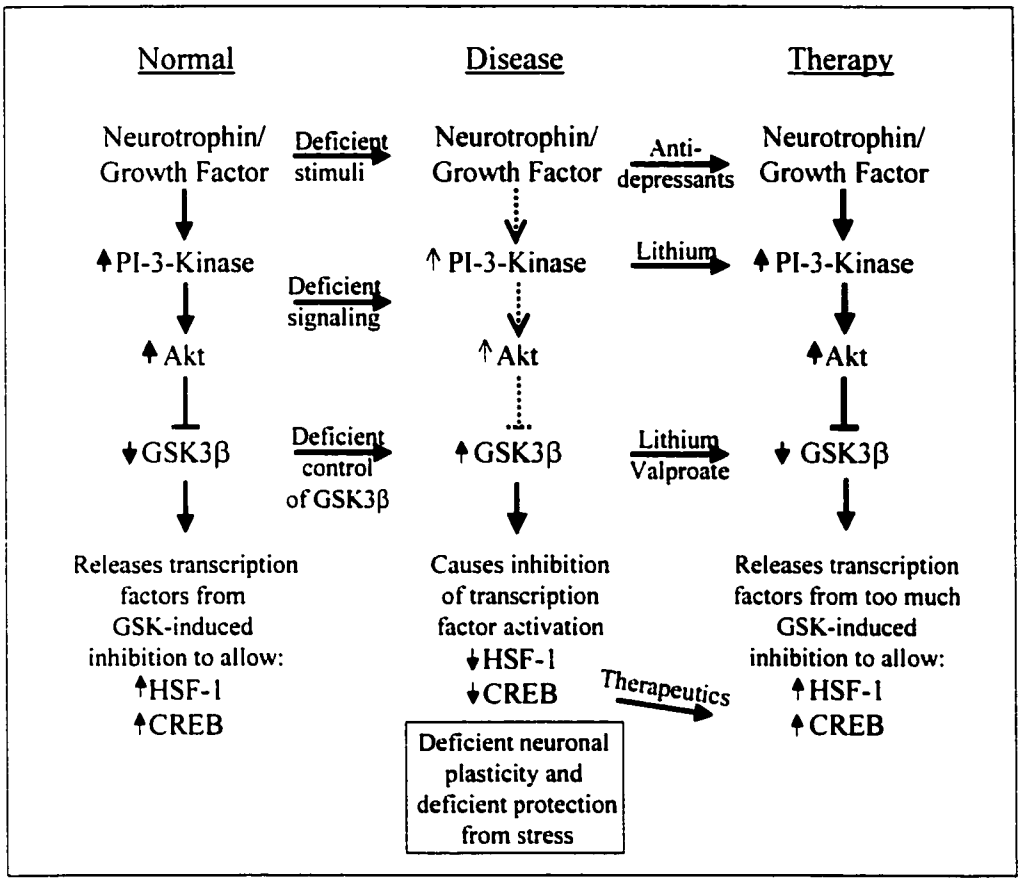


**FIG. 8.** Potential involvement of GSK3β in mood disorders and actions of therapeutic agents. Deficits in the inhibitory control of GSK3β derived from any of the signaling cascades that regulate GSK3β activity or expression may result in excessive GSK3β-mediated inhibition of transcription factors. There are multiple targets for therapeutic agents to enhance the inhibition of GSK3β and thereby facilitate transcription factor activation which may contribute to recovery, mood stabilization, neuronal plasticity, and cell survival.

signals, lithium may facilitate mood stabilization. Additionally, a component of lithium's beneficial effects may stem from its broad neuroprotective properties, an action that we propose results, in part, from its inhibition of GSK3β, which itself has broad inhibitory effects on survival-promoting transcription factors.

*Alzheimer's disease*

One of the most notable developments in research concerning GSK3β is that solid evidence directly links GSK3β to many of the major neuropathological mechanisms that have been identified to be associated with Alzheimer's disease. These include interactions between GSK3β and components of the plaque-producing amyloid system associated with Alzheimer's disease; the participation of GSK3β in phosphorylating the microtubule-binding protein tau, which may contribute to the formation of neurofibrillary tangles in Alzheimer's disease; interactions of GSK3β with presenilin and other Alzheimer's disease-associated proteins; and the facilitation of apoptosis by GSK3β, each of which is discussed below. These actions are summarized in Fig. 9.

GSK3β levels or activity have been measured in brain tissue in several studies of Alzheimer's disease. Initial examination of the levels of GSK3β in several tissues from rats revealed that the brain contained the highest levels (Woodgett, 1990). Increased levels of GSK3β have been found in Alzheimer's disease, compared with nondiseased, human brain, and immunohistochemical measurements have located GSK3β associated with neurofibrillary tangles in Alzheimer's disease brain (Yamaguchi et al., 1996; Imahori and Uchida, 1997; Pei et al., 1997, 1999). Additionally, active GSK3β was found to be accumulated in pretangle neurons (Pei et al., 1999). Taken together, these

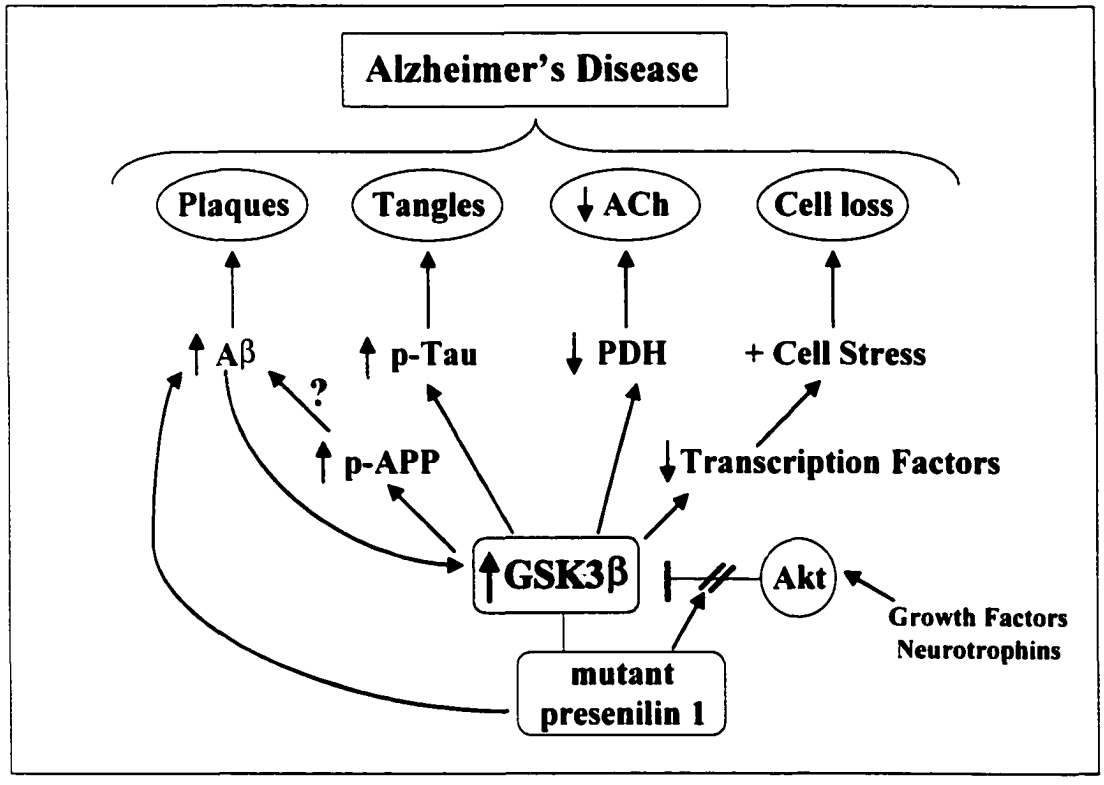


**FIG. 9.** GSK3β is associated with neuropathological mechanisms involved in Alzheimer's disease. GSK3β is activated by Aβ and phosphorylates APP. GSK3β phosphorylates tau, which might contribute to neurofibrillary tangle formation. GSK3β phosphorylates PDH, resulting in decreased acetylcholine synthesis. GSK3β inhibits cell several survival-promoting transcription factors which, in concert with a wide variety of cell stressors, facilitates neuronal death. Taken together, these actions suggest that GSK3β may contribute to the neuropathologies characteristic of Alzheimer's disease.

studies provide tantalizing evidence that alterations in the control of GSK3β may occur in Alzheimer's disease brain, a possibility that should be explored in further investigations.

Evidence from several studies has associated GSK3β with the amyloid plaque neuropathology of Alzheimer's disease through its interactions with the amyloid precursor protein, the Aβ peptide product of the proteolysis of amyloid precursor protein, and the inhibition by Aβ of pyruvate dehydrogenase (reviewed in Imahori and Uchida, 1997). GSK3β phosphorylates the amyloid precursor protein (Aplin et al., 1996, 1997), and the neurotoxic Aβ peptide derived from cleavage of the amyloid precursor protein activates GSK3β (Takashima et al., 1995). Aβ-induced activation of GSK3β was reported to lead to phosphorylation of tau (Takashima et al., 1996, 1998a), which may contribute to the accumulation of hyperphosphorylated tau in Alzheimer's disease. Furthermore, Aβ-induced neurotoxicity was reduced by antisense GSK3β oligonucleotides (Takashima et al., 1993) and by the GSK3β inhibitor lithium (Alvarez et al., 1999; Wei et al., 2000). Additionally, Aβ-activated GSK3β phosphorylated and inactivated pyruvate dehydrogenase (Hoshi et al., 1996), resulting in reduced synthesis of acetylcholine as a result of depletion of its precursor acetyl coenzyme A (Hoshi et al., 1997; Imahori and Uchida, 1997). These observations tie GSK3β to the well-documented finding that there is a severe loss of cholinergic signaling in Alzheimer's disease. Thus, Aβ may contribute to activation of GSK3β in Alzheimer's disease brain which, in turn, contributes to Aβ-induced neurotoxicity. This may, in turn, both reduce acetylcholine synthesis by inhibiting pyruvate dehydrogenase and increase the phosphorylation of tau (Imahori and Uchida, 1997).

Neurofibrillary tangles constitute one of the neuropathological hallmarks of Alzheimer's disease. These are composed primarily of tau, and tau in these tangles is abnormally hyperphosphorylated. There is considerable evidence that GSK3β contributes to the phosphorylation of tau, and there is speculation that GSK3β may contribute to the abnormal hyperphosphorylation of tau observed in neurofibrillary tangles (as discussed in two comprehensive reviews, Johnson and Jenkins, 1996; Johnson and Hartigan, 1998). The hyperphosphorylation state of tau in Alzheimer's disease may cause dissociation of tau from microtubules, abnormal localization and/or accumulation of tau, and contribute to aggregation of tau, but it is also possible that the abnormal hyperphosphorylation of tau occurs after tau aggregation has been initiated by other mechanisms. These questions remain the topics of intense investigation. It is of considerable interest that GSK3β is able to phosphorylate some of the same sites on tau that are abnormally hyperphosphorylated in Alzheimer's disease brain, although a cause and effect relationship has not been established. At least a portion of the GSK3β-mediated phosphorylation of tau may be hierarchical, as prior phosphorylation of tau by cdk5 (also called tau protein kinase II) enhanced the subsequent phosphorylation of tau by GSK3β (Ishiguro et al., 1992; Sengupta et al., 1997). Overall, it is evident that tau is the major component of neurofibrillary tangles and is abnormally hyperphosphorylated in Alzheimer's disease brain, and that GSK3β can phosphorylate several sites on tau. While it is intriguing to consider the possibilities that phosphorylation of tau by GSK3β is abnormally induced in Alzheimer's disease, and that this contributes to neurofibrillary tangle formation, these relationships remain to be tested in an unequivocal manner.

Very intriguing, but still incompletely understood, associations between GSK3β and presenilin proteins have been identified by investigators interested in the neurodegenerative mechanisms that cause Alzheimer's disease. Mutations in the genes for presenilin 1 and 2 account for most of the cases of familial Alzheimer's disease, although the large majority of Alzheimer's disease patients are classified as sporadic with no known genetic cause (Hardy, 1997). Mutations in the presenilin 1 gene cause an autosomal dominant inheritance of early onset Alzheimer's disease with 100% penetrance in causing Alzheimer's disease (Haass and De Strooper, 1999). Thus, there is widespread interest in identifying the functions of presenilins and how mutations contribute to neuronal dysfunction and degeneration. One possibility is suggested by the finding that Alzheimer's disease-associated mutations in presenilin 1 facilitate apoptosis in several model systems, such as apoptosis caused by the Alzheimer's disease Aβ-peptide (Guo et al., 1997; Kovacs et al., 1999; Tanii et al., 2000; but for a contrasting viewpoint see Bursztajn et al., 1998). However, the mechanisms by which mutations in presenilin genes facilitate apoptosis and contribute to Alzheimer's disease remain elusive.

Several investigators recently reported that presenilin 1 binds to GSK3β (Takashima et al., 1998b; Kang et al., 1999; Gantier et al., 2000). Takashima et al (1998b) first reported a direct interaction between presenilin 1 and GSK3β by showing that GSK3β was detectable in presenilin 1 immunoprecipitates from human brain. The same immunoprecipitates also demonstrated positive immunoreactivity for tau, suggesting that presenilin 1 can facilitate the colocalization of GSK3β and tau and, thus, may facilitate GSK3β-mediated phosphorylation of tau. As noted previously (Takashima et al., 1998b; Kang et al., 1999), presenilin 1 also binds other substrates of GSK3β in

addition to tau, including δ-catenin (Zhou et al., 1997), β-catenin (Yu et al., 1998; Kang et al., 1999), and amyloid precursor protein (Aplin et al., 1996); so, presenilin 1 may act as a scaffold for bringing GSK3β into close proximity with these substrates that have critical roles in Alzheimer's disease and cell survival/apoptotic mechanisms.

In contrast to the consistent findings that presenilin 1 binds GSK3β, contradictory results have been published concerning how Alzheimer's disease-associated mutations of presenilin 1 affect this interaction. Takashima et al. (1998b) reported that expression of the C263R and P264L mutants of presenilin 1 in COS-7 cells caused a 3-fold increase in the amount of GSK3β that coimmunoprecipitated with presenilin 1, compared with wild-type presenilin 1. An opposite finding was reported by Kang et al. (1999), who demonstrated that the M146L and ΔX9 presenilin 1 mutants bound GSK3β less than wild-type presenilin 1. Gantier et al. (2000) further showed that the L392V mutation of presenilin 1 decreased its affinity for GSK3β relative to wild-type presenilin 1. The contradictory findings in these studies could be ascribed to the different presenilin 1 mutants and different methods used in each study, or they may indicate that any perturbation of the interactions between presenilin 1 and GSK3β, whether it be increased or decreased association, impairs the normal control or function of the proteins. These conflicting data leave open the question of what role altered association of GSK3β with mutant presenilin 1 contributes to the early onset Alzheimer's disease associated with mutations in the presenilin 1 gene, but all of the studies are in agreement that wild-type presenilin 1 binds GSK3β and, thus, may be an important modulator of the actions of GSK3β.

In addition to directly binding GSK3β, there is also evidence that presenilin 1 is capable of modulating its activity by regulating Akt. Weihl et al. (1999) reported that transient transfection of mutant presenilin 1 was associated with decreased Akt activity compared with wild-type presenilin 1. Cells expressing mutant presenilin 1 also contained lower levels of phosphorylated Ser-9 GSK3β, in accordance with the lower levels of active Akt, which mediates phosphorylation of Ser-9 of GSK3β, and higher levels of phosphorylated Tyr-216 GSK3β, an active form of the kinase (Weihl et al., 1999). These data suggest that mutant presenilin 1 may up-regulate GSK3β activity by perturbing its inhibitory control by Akt. Such an action could contribute to increased deleterious actions of GSK3β, such as increased phosphorylation of tau and neuronal apoptosis, conditions associated with Alzheimer's disease.

Overall, these studies provide increasing evidence of interactions between presenilin 1 and GSK3β and demonstrate that mutant forms of presenilin 1 can modify the activity and function of GSK3β. It remains unclear exactly how presenilin 1 mutants alter GSK3β function, but mutant forms of presenilin 1 may indirectly both modify the control of GSK3β activity and alter its intracellular localization and proximity to certain substrates. These findings raise the possibility that the increased vulnerability to apoptosis rendered by mutations of presenilin 1 may be linked to the altered control of GSK3β, which itself has been shown to play a crucial role in the control of apoptosis (Pap and Cooper, 1998; Bijur et al., 2000).

Taking into account the multiple potential links between hyperactive GSK3β and Alzheimer's disease, consideration should be given to the possibility that transcription factors affected by GSK3β may be abnormally regulated in Alzheimer's disease. As noted

above, evidence for hyperactive GSK3β associated with Alzheimer's disease has been obtained in assessments of GSK3β localization in Alzheimer's disease and the interactions of GSK3β with Aβ, tau, and presenilin 1. Taken together, this evidence raises the possibility that inhibitory control of GSK3β has been compromised in afflicted neurons. This condition can be predicted to result in GSK3β-mediated impaired activation of transcription factors. It is notable that several of the GSK3β-inhibited transcription factors are critical components of the mechanisms cells use to survive stress and potentially lethal insults. These factors include HSF-1, NFκB, CREB, and others, impairments of which are well documented to increase the susceptibility of cells to toxic insults. Thus, not only is GSK3β associated with the major neuropathological markers of Alzheimer's disease, but dysfunctional control of GSK3β may further compromise the capabilities of cells to respond adequately to cell stress through GSK3β's inhibitory influences on cell survival-promoting transcription factors.

Taken together, there is a surprisingly large number of interactions between GSK3β and key cellular components related to the neuropathology of Alzheimer's disease, as summarized in Fig. 9. These include interactions between GSK3β and the amyloid precursor protein; the Aβ peptide; the metabolic pathway leading to acetylcholine synthesis; tau protein, which is the primary constituent of neurofibrillary tangles; and the presenilins, which are mutated in many cases of familial Alzheimer's disease. Furthermore, recent studies have revealed that GSK3β can contribute to apoptotic signaling activities. The cumulative impression given by these diverse findings is that the control of GSK3β, or its abnormal control, may be a focal point integrating the

diverse neuropathologies associated with Alzheimer's disease, a proposition that certainly warrants further investigation.

*Cell survival and apoptosis*

In the last few years, GSK3β has rapidly become recognized as an important modulator of apoptosis. Among the intriguing links between GSK3β and cell survival that have been identified are the findings that activation of GSK3β is linked directly to increased neuronal apoptosis; that GSK3β down-regulates the activities of several transcription factors that are critical promoters of cell survival, such as HSF-1 and CREB; and that lithium, which inhibits GSK3β, protects neurons from the lethality of a wide variety of toxic insults. These actions of GSK3β may be associated with its links to neurodegenerative disorders, such as Alzheimer's disease, and also raise the specter of cell loss mediated by hyperactive GSK3β, and protection by lithium, contributing to bipolar disorder.

GSK3β has been directly linked to neuronal apoptosis in several recent studies. The first evidence that activation of GSK3β could be associated with neuronal apoptosis was reported by Takashima et al. (1993), who found that application of antisense oligonucleotides to reduce GSK3β levels protected cells from Aβ-induced neurotoxicity. More direct evidence was provided by Pap and Cooper (1998), who found that transient overexpression of active GSK3β in PC12 and Rat-1 cells was sufficient to induce apoptosis. They also found that expression of catalytically inactive GSK3β reduced apoptosis induced by an inhibitor of PI3K and that expression of a dominant-negative mutant of p53 attenuated GSK3β-induced apoptosis. These important findings revealed

for the first time that GSK3β is one of the key targets of the well-established anti-apoptotic signaling mediated by the PI3K/Akt pathway (Pap and Cooper, 1998). Bijur et al. (2000) extended those findings by showing that relatively low levels of overexpression of GSK3β, which alone did not induce apoptosis, greatly facilitated proapoptotic signaling activities. These findings emphasized the importance of cellular signaling mechanisms that maintain inhibitory control of GSK3β activity. Additionally, control of overactive GSK3β was shown be attainable pharmacologically with the GSK3β-inhibiting drug lithium (Bijur et al., 2000). Thus, apoptosis associated with hyperactive GSK3β was controlled with lithium, results that provide direct evidence of a site of action contributing to the neuroprotectant properties of lithium.

Withdrawal of trophic factors from cultured neurons is one of the most widely studied models of apoptosis, and GSK3β activity was recently implicated in this process (Hetman et al., 2000). Serum withdrawal from cultured cortical neurons caused apoptosis, along with decreased Akt activity and increased GSK3β activity. The direct involvement of GSK3β in the development of apoptosis was demonstrated when apoptosis was attenuated by treatments that reduced the activation of GSK3β (Hetman et al., 2000). On the other hand, Crowder and Freeman (2000) reported that apoptosis induced by NGF-withdrawal was independent of GSK3β, although GSK3β was necessary for neuronal death caused by inhibition of PI3K. The potential relevance of GSK3β-associated cell death to a specific neurodegenerative condition was found by Maggirwar et al. (1999). They reported that the human immunodeficiency virus type 1 regulatory protein Tat activates GSK3β and induces apoptosis in cerebellar granule neurons. Their results suggested that Tat activated platelet-activating factor receptors,

which increased GSK3β activity and thereby caused cell death, an outcome blocked by the GSK3β inhibitor lithium (Maggirwar et al., 1999). Recently, Bhat et al. (2000) reported that several proapoptotic stimuli increased the activity and tyrosine-216 phosphorylation of GSK3β and that inhibition of GSK3β reduced cell death, providing further evidence for a critical role of GSK3β in apoptotic signaling. Taken together, this handful of recent studies leaves little doubt that GSK3β is a critical intermediary in a variety of apoptotic signaling pathways and that the inhibitory control of GSK3β by anti-apoptotic signaling systems, such as the PI3K-Akt pathway, is an important facet of their mechanisms used to promote cell survival. Activation of Akt, which inhibits GSK3β, is one of the most well-characterized cell-survival signaling pathways. Although the protective effect of Akt has been linked to a number of antiapoptotic mechanisms, such as inactivation of the proapoptotic caspase-9 (Cardone et al., 1998) and Bad (Datta et al., 1997; del Peso et al., 1997), it now appears that its inhibition of GSK3β also contributes a critical component to the protective effects attained by activation of Akt.

The finding that lithium can protect neurons from apoptosis by inhibiting the activity of GSK3β (Bijur et al., 2000) provides an important clue toward solving one of the most intriguing properties of lithium: that it is able to provide neuroprotection from a wide variety of insults (reviewed in Jope, 1999a). A rapidly burgeoning body of literature has provided ample evidence that lithium is a neuroprotectant. Table 2 summarizes the multiple neurotoxic conditions that have been reported to be ameliorated by lithium treatment. Although few of these studies directly linked the inhibitory effect of lithium on GSK3β to lithium's neuroprotection, emerging data summarized in previous sections of this article indicate that this is likely to be an important mechanism

**Table 2.** Lithium provides neurons protection against many insults

| Toxic insult | Reference |
|---|---|
| Serum and NGF deprivation, PC12 cells | Volonte and Rukenstein, 1993 |
| Low $K^+$, cerebellar granule cells | D'Mello et al., 1994 |
| Ouabain-induced cell death, PC12 and SH-SY5Y cells | Li et al., 1994 |
| Primary rat cortical and cerebellar cultures | Volonte et al., 1994 |
| Radiation-induced apoptosis, cerebellar granule cells | Inouye et al., 1995 |
| Ibotenate lesions, rat basal forebrain | Pascual and Gonzalez, 1995 |
| Low $K^+$, cerebellar granule cells | Grignon et al., 1996 |
| Ceramide-induced apoptosis, cerebellar granule cells | Centeno et al., 1998 |
| Focal ischemia, rat cortex | Nonaka and Chuang, 1998 |
| Glutamate excitotoxicity, cerebellar granule cells | Nonaka et al., 1998a |
| Drug-induced apoptosis, rat cerebellar granule cells | Nonaka et al., 1998b |
| β-amyloid-induced death, primary rat cortical cultures | Alvarez et al., 1999 |
| Ibotenic lesions, rat cortex | Arendt et al., 1999 |
| $Na^+$ depletion, cerebellar granule cells | Khodorov et al., 1999 |
| Tat-induced apoptosis, cerebellar granule cells | Maggirwar et al., 1999 |
| Low $K^+$, cerebellar granule cells | Mora et al., 1999 |
| NGF withdrawal, PC12 cells | Bhat et al., 2000 |
| Staurosporine and heat shock, SH-SY5Y cells | Bijur et al., 2000 |
| Valinomycin-induced death, SH-SY5Y cells | Li and El-Mallakh, 2000 |
| β-amyloid-induced death, PC12 cells | Wei et al., 2000 |

contributing to the protection furnished by lithium. These findings have raised two intriguing questions regarding the therapeutic actions of lithium. One of these is the question of whether the neuroprotective effect of lithium is directly connected with its action as a mood stabilizer. There is a growing amount of evidence for cell loss in mood disorders, and this raises the critical questions of whether lithium lessens this loss, whether the anticonvulsants that are also being applied as mood stabilizers are equally neuroprotectant, and what role neuroprotection plays in the therapeutic actions of these drugs. Especially intriguing is the recent hypothesis (Jacobs et al., 2000) that mood stabilizers promote the survival of newly formed neurons in adult brain (neurogenesis), an action we propose is likely promoted by inhibition of GSK3β. Second, the neuroprotectant action of lithium has raised the question of whether it would ameliorate cell loss associated with neurodegenerative diseases. These questions are currently being

addressed in many studies, the results of which should not only increase our understanding of the actions of lithium, but may also provide new methods for therapeutically lessening neuronal loss associated with degenerative conditions.

The mechanisms by which GSK3β facilitates apoptosis have yet to be identified. However, several potential contributory mechanisms are evident from the known actions of GSK3β reviewed above. For example, GSK3β-induced phosphorylation of tau may destabilize microtubules to contribute to cytoskeletal collapse associated with apoptosis, and GSK3β-mediated phosphorylation of pyruvate dehydrogenase may impair Krebs cycle activity. These are but two targets exemplifying the conclusion that multiple known targets of GSK3β have the potential for contributing to its proapoptotic actions. However, we are especially intrigued by the mechanisms involved with its regulatory effects on the activation of transcription factors. GSK3β inhibits the activation of many transcription factors, several of which are well known to promote cell survival. Thus, we propose that GSK3β-induced inhibition of these transcription factors contributes to its proapoptotic actions and, conversely, that lithium's neuroprotectant actions stem at least in part from its facilitory effects on transcription factor activation resulting from its inhibition of GSK3β. We suggest that this diversity of affected transcription factors associated with cell survival that are affected by GSK3β and lithium likely is a major reason why these modulate such a diverse array of cell death-associated signaling pathways. Several of the affected transcription factors are discussed below.

Two of the transcription factors that are likely candidates as important targets for GSK3β, and consequently lithium, in relation to cell survival are HSF-1 and CREB. The inhibitory effect of GSK3β on stress-induced activation of HSF-1 is likely to impede cell

survival (Bijur and Jope, 2000). Upon activation, HSF-1 induces the expression of heat shock proteins that detect and chaperone denatured and misfolded proteins. Many studies have shown that blockade of heat shock protein expression facilitates cell death and that overexpression of heat shock proteins attenuates stress-induced cell death (Kiang and Tsokos, 1998; Morimoto, 1998). Thus, inadequate inhibition of GSK3β activity is likely to impair the expression of heat shock proteins through GSK3β's inhibition of HSF-1 activation, as recently reported (Bijur and Jope, 2000), and thus increase cellular susceptibility to stress-induced death. Likewise, inhibition of the activity of CREB also may be contribute to the proapoptotic effects of GSK3β, since it has recently become clear that CREB is a key promoter of neuronal survival (Walton and Dragunow, 2000). Reduction of CREB activity has been reported to facilitate apoptosis (Jean et al., 1998; Walton et al., 1999), and CREB upregulates the expression of the antiapoptotic protein bcl-2 (Ji et al., 1996; Wilson et al., 1996; Pugazhenthi et al., 1999; Riccio et al., 1999; Pugazhenthi et al., 2000). Thus, reduction of CREB activity by GSK3β as recently reported (Grimes and Jope, 2000) may contribute to increased susceptibility to apoptosis engendered by GSK3β.

Similarly, modulation by GSK3β, and lithium, of several other transcription factors such as NFκB, AP-1, β-catenin, and myc likely contribute to modulation of cell survival under certain conditions. Each of these can promote cell survival in certain conditions, and each is inhibited by GSK3β. For example, although the modulatory effect of GSK3β on activation of NFκB remains unclear, there is much evidence that NFκB is an important promoter of neuronal survival (Mattson, 1997), and Bournat et al. (2000) reported evidence that activation of NFκB in association with inhibition of GSK3β

promoted survival of PC12 cells. Overall, consideration of these multiple transcription factors that are modulators of cell survival and are inhibited by GSK3β indicates that impaired inhibitory control of GSK3β, or uncontrolled activation of GSK3β, initiates an array of changes in transcription factor activities that can contribute to the apoptotic process. It seems likely that the importance of particular transcription factors in this regard will vary depending on cell type and apoptotic mechanism. This diversity of transcription factors also suggests a rationale for the highly diverse apoptotic stimuli that have been shown to be attenuated by lithium (Table 2).

Among the several possible mechanisms that might contribute to the pro-apoptotic action of GSK3β and the neuroprotective action of lithium, Fig. 10 illustrates one mechanism we find especially intriguing. Fig. 10A describes the pro-apoptotic mechanism of action of GSK3β. Although the previous literature was contradictory concerning the functional interactions between GSK3β and CREB, we have demonstrated quite clearly that GSK3β activity inhibits CREB (Grimes and Jope, 2000). Much data also has shown that GSK3β reduces the levels, and therefore transcriptional activity, of other CBP-binding transcription factors, including β-catenin and other transcription factors not represented in the figure. Both CREB (Giordano andAvantaggiati, 1999) and β-catenin (Waltzer and Bienz, 1998; Hecht et al., 2000; Takemaru and Moon, 2000) bind the coactivator CBP; therefore, inhibition of their activities by GSK3β will effectively release CBP to bind to other transcription factors. A key recent finding by Lee et al. (2000) provides one of the cornerstones of this proapoptotic scheme. They discovered that CBP released from CREB could be bound by p53 and facilitate p53-mediated transcription (Lee et al., 2000), which is well known to lead to apoptosis. Thus, activation of GSK3β functionally

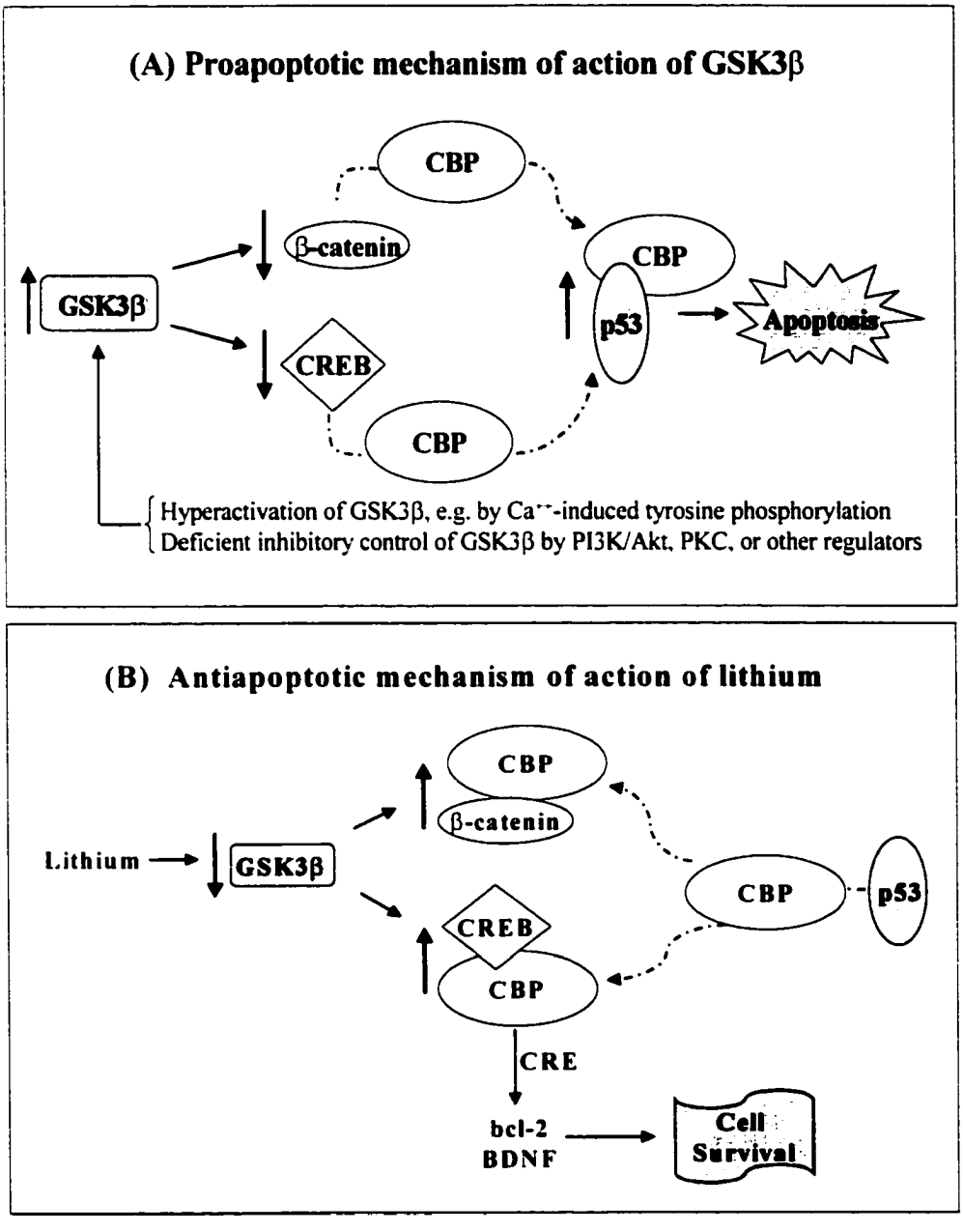


**FIG. 10.** GSK3β-linked proapoptotic signaling pathways and protective mechanism of lithium. **A:** The inhibitory effects of GSK3β on survival-promoting transcription factors, including CREB, β-catenin, and others not depicted, may release CBP to facilitate its interaction with p53 and the subsequent facilitation of apoptosis. **B:** Lithium inhibits GSK3β, allowing enhanced activity and CBP binding of survival-promoting transcription factors, including CREB, β-catenin, and others not depicted, thus drawing CBP away from p53 to reduce apoptosis. Arrows indicate changes in functional activity.

inhibits CREB, β-catenin, and other survival-promoting transcription factors that bind CBP and, by releasing CBP, contribute to activation of p53, and apoptosis ensues.

Additional evidence for this scheme comes from recent reports that CREB has antiapoptotic actions (Jean et al., 1998; Bonni et al., 1999; Riccio et al., 1999; Walton et al., 1999), and the finding by Pap and Cooper (1998) that GSK3β-induced apoptosis was inhibited by expression of a dominant-negative p53. Therefore, with the recent clarification of the inhibitory effect of GSK3β on CREB activation, evidence supporting each of the individual steps of the pro-apoptotic scheme shown in Fig. 10A has been reported.

One of the neuroprotective mechanisms of action of lithium follows from this pro-apoptotic mechanism of action of GSK3β (Fig. 10B). We propose that as a consequence of lithium's inhibition of GSK3β, the activity of CREB is increased (Grimes and Jope, 2000), as well as the activity of other CBP-binding transcription factors, such as β-catenin. Lithium-induced enhanced activation of these CBP-binding transcription factors would be predicted to reduce the CBP available to bind p53 and thereby reduce the activity of p53 and its proapoptotic function. In accordance with this scheme, lithium has previously been shown to reduce p53 (Chen and Chuang, 1999; Lu et al., 1999). It is notable, that among its actions, p53 acts as a suppressor of bcl-2 expression, thus providing another point of balance between the antiapoptotic actions of CREB, which can increase bcl-2 expression (Wilson et al., 1996), and the proapoptotic effect of p53 (reviewed in Finkbeiner, 2000). Previous studies have reported that lithium leads to increased levels of bcl-2 (Chen et al., 1999b; Chen and Chuang, 1999). In addition to an antiapoptotic outcome as a result of reduced p53 activity, increased CREB activity may

also increase the expression of other antiapoptotic proteins such as BDNF (Tao et al., 1998). Interestingly, increased expression of BDNF has also been proposed to be an important therapeutic response to treatment with antidepressants (Duman et al., 1997), providing an overlap between the actions of the antibipolar agent lithium and antidepressants. Finally, the inhibition of GSK3β by lithium should act in concert with growth factors whose signaling down-regulates GSK3β activity (e.g., through PI3K/Akt signaling) to promote neurogenesis. Overall, this mechanism that we propose for the neuroprotective action of lithium incorporates a wide variety of disparate findings into a single scheme (Fig. 10B).

There are two facets of the schemes depicted in Fig. 10 that we find especially noteworthy. These are the breadth of the antiapoptotic mechanisms bolstered by lithium and the relatively modest changes likely to be induced on these by therapeutic concentrations of lithium. The breadth of lithium's effects, derived from the multiple transcription factors regulated by GSK3β, likely contributes to the large variety of toxic insults that lithium is able to counter. By enhancing many antiapoptotic signaling mechanisms, the neuroprotective actions of lithium are not limited to a select few types of cell stress, but instead enables lithium to provide protection from a remarkably varied number of neurotoxic insults that have been reported to be ameliorated by lithium (Table 2). On the other hand, the protection that lithium affords may be limited to mild-to-moderate toxic insults. This proposal is based on the evidence that at the therapeutic concentration of 1 mM, lithium will inhibit GSK3β by less than 50%. This degree of inhibition should shift the balance of signaling systems toward the enhanced production

of antiapoptotic agents that are associated with partial inhibition of GSK3β, and thus facilitate activation of the associated transcription factors.

## SUMMARY

GSK3β is a fascinating enzyme with an astoundingly diverse number of actions in intracellular signaling systems. We find especially intriguing the evidence indicating that GSK3β has the key role of a "master regulator" over a broad array of transcription factors. As this article has noted, many of the transcription factors activated when GSK3β is inhibited contribute to cell proliferation and survival. Thus, GSK3β appears to be a gatekeeper, maintaining cells at a regulated rate of proliferation and in a condition where cell death pathways can be employed if needed. It appears that the consequences associated with loss of control of GSK3β could be devastating, with overactive GSK3β increasing vulnerability to potentially lethal insults and too little GSK3β allowing uncontrolled proliferation. Among the possible diseases that may be associated with dysfunctional regulation of GSK3β, Alzheimer's disease is especially intriguing. This is so because GSK3β has been directly linked to all of the key components of Alzheimer's disease that have been identified, including amyloid, tangles, presenilins, and cholinergic function. Equally intriguing is the selective inhibitory effect on GSK3β exerted by lithium, the primary therapeutic treatment for bipolar disorder. Whether lithium's inhibition of GSK3β contributes to its mood-stabilizing properties is a difficult problem to address directly, and so it remains an intriguing subject for further investigation. Nevertheless, this action of lithium has stimulated investigations of lithium's actions on substrates of GSK3β as a first step toward identifying effects of lithium that may be

related to its therapeutic effects. Finally, GSK3β may affect both Alzheimer's disease and bipolar disorder through its proapoptotic actions, a capability that may play a fundamental role in development and cell survival, and which has led us to suggest that lithium's broad cell survival-promoting effects may be a consequence of its inhibition of GSK3β. Overall, GSK3β has far-reaching effects on cellular functions, the consequences of which are only beginning to be recognized.

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## REFERENCES

Alberts A. S., Arias J., Hagiwara M., Montminy M. R., and Feramisco J. R. (1994) Recombinant cyclic AMP response element binding protein (CREB) phosphorylated on Ser-133 is transcriptionally active upon its introduction into fibroblast nuclei. *J. Biol. Chem.* **269**, 7623-7630.

Alessi D. R., Andjelkovic M., Caudwell, B., Cron P., Morrice N., Cohen P., and Hemmings, B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**, 6541-6551.

Alvarez G., Munoz-Montano J. R., Satrustegui J., Avila J., Bogonez E., and Diaz-Nido J. (1999) Lithium protects cultured neurons against β-amyloid-induced neurodegeneration. *FEBS Lett.* **453**, 260-264.

Angel P., Imagawa M., Stein B., Imbra R. J., Rahmsdorf H. J., Jonat C., Herrlich P., and Karin M. (1987) Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* **49**, 729-739.

Aplin A. E., Gibb G. M., Jacobsen J. S., Gallo J.M., and Anderton B.H. (1996) In vitro phosphorylation of the cytoplasmic domain of the amyloid precursor protein by glycogen synthase kinase-3β. *J. Neurochem.* **67**, 699-707.

Aplin A. E., Jacobsen J. S., Anderton B. H., and Gallo J. M. (1997) Effect of increased glycogen synthase kinase-3 activity upon the maturation of the amyloid precursor protein in transfected cells. *Neuroreport* **8**, 639-643.

Arendt T., Lehmann K., Seeger G., and Gartner U. (1999) Synergistic effects of tetrahydroaminoacridine and lithium on cholinergic function after excitotoxic basal forebrain lesions in rat. *Pharmacopsychiatry* **32**, 242-247.

Arias J., Alberts A. S., Brindle P., Claret F. X., Smeal T., Karin M., Feramisco J., and Montminy M. R. (1994) Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**, 226-229.

Asghari V., Wang J. F., Reiach J. S., and Young L. T. (1998) Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. *Mol. Brain Res.* **58**, 95-102.

Baeuerle P. A. and Baltimore D. (1988) IκB: a specific inhibitor of the NF-κB transcription factor. *Science* **242**, 540-546.

Baeuerle P. A. and Baltimore D. (1989) A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. *Genes Dev.* **3**, 1689-1698.

Baeuerle P. A. and Henkel T. (1994) Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* **12**, 141-179.

Baldwin A. S. (1996) The NFκB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649-681.

Beals C. R., Sheridan C. M., Turck C. W. Gardner, P., and Crabtree G. R. (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275**, 1930-1933.

Beg A. A., Ruben S. M., Scheinman R. I., Haskill S., Rosen C. A., and Baldwin A. S. (1992) IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. *Genes Dev.* **6**, 1899-1913.

Beg A. A., Finco T. S., Nantermet P. V., and Baldwin A. S. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. *Mol. Cell. Biol.* **13**, 3301-3310.

Behrens J., von Kries J. P., Kuhl M., Bruhn L., Wedlich D., Grosschedl R., and Birchmeier W. (1996) Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* **382**, 638-642.

Behrens J., Jerchow B. A., Wurtele M., Grimm, J., Asbrand C., Wirtz R., Kuhl M., Wedlich D., and Birchmeier W. (1998) Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β. *Science* **280**, 596-599.

Berling B., Wille H., Roll B., Mandelkow E. M., Garner C., and Mandelkow E. (1994) Phosphorylation of microtubule-associated proteins MAP2a,b and MAP2c at Ser136 by proline-directed kinases in vivo and in vitro. *Eur. J. Cell Biol.* **64**, 120-30

Bevilaqua L. R., Cammarota M., Paratcha G., de Stein M. L., Izquierdo I., and Medina J. H. (1999) Experience-dependent increase in cAMP-responsive element binding protein in synaptic and nonsynaptic mitochondria of the rat hippocampus. *Eur. J. Neurosci.* **11**, 3753-3756.

Bhat R. V., Shanley J., Correll M. P., Fieles W. E., Keith R. A., Scott C. W., and Lee C. M. (2000) Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3β in cellular and animal models of neuronal degeneration. *Proc. Natl. Acad. Sci. USA* **97**, 11074-11079.

Bijur G. N., De Sarno P., and Jope R. S. (2000) Glycogen synthase kinase-3β facilitates staurosporine- and heat shock-induced apoptosis: protection by lithium. *J. Biol. Chem.* **275**, 7583-7590.

Bijur G. N. and Jope R. S. (2000) Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3β in the regulation of HSF-1 activity. *J. Neurochem.* **75**, 2401-2408.

Blackwell T. K., Kretsner L., Blackwood E. M., Eisenman R. N., and Weintraub H. (1990) Sequence-specific DNA binding by the c-Myc protein. *Science* **250**, 1149-1151.

Blackwood E. M. and Eisenman R. N. (1991) Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **251**, 1211-1217.

Blair A. S., Hajduch E., Litherland G. J., and Hundal H. S. (1999) Regulation of glucose transport and glycogen synthesis in L6 muscle cells during oxidative stress. Evidence for cross-talk between the insulin and SAPK2/p38 mitogen-activated protein kinase signaling pathways. *J. Biol. Chem.* **274**, 36293-36299.

Blendy J. A., Kaestner K. H., Schmid W., Gass P., and Schutz G. (1996) Targeting of the CREB gene leads to up-regulation of a novel CREB mRNA isoform. *EMBO J.* **15**, 1098-1106.

Bonni A., Brunet A., West A. E., Datta S. R., Takasu M. A., and Greenberg M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**, 1358-1362.

Bournat J. C., Brown A. M. C., and Soler A. P. (2000) Wnt-1 dependent activation of the survival factor NF-κB in PC12 cells. *J. Neurosci. Res.* **61**, 21-32.

Bourtchuladze R., Frenguelli B., Blendy G., Cioffi D., Chutz G., and Silva, A. J. (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* **79**, 59-68.

Boyle W. J., Smeal T., Defize L. H., Angel P., Woodgett J. R., Karin M., and Hunter T. (1991) Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* **64**, 573-584.

Brindle P. K. and Montminy M. R. (1992) The CREB family of transcription activators. *Curr. Opin. Genet. Dev.* **2**, 199-204.

Bullock B. P. and Habener J. F. (1998) Phosphorylation of the cAMP response element binding protein CREB by cAMP-dependent protein kinase A and glycogen synthase kinase-3 alters DNA-binding affinity, conformation, and increases net charge. *Biochemistry* **37**, 3795-3809.

Bursztajn S., DeSouza R., McPhie D. L., Berman S. A., Shioi J., Robakis N. K., and Neve R.L. (1998) Overexpression in neurons of human presenilin-1 or a presenilin-1 familial Alzheimer disease mutant does not enhance apoptosis. *J. Neurosci.* **18**, 9790-9799.

Cardone M. H., Roy N., Stennicke H. R., Salvesen G. S., Franke T. F., Stanbridge E, Frisch S., and Reed J. C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282**, 1318-1321.

Castro A. and Martinez A. (2000) Inhibition of tau phosphorylation: a new therapeutic strategy for the treatment of Alzheimer's disease and other neurodegenerative disorders. *Exp. Opin. Ther. Patients* **10**, 1519-1527.

Centeno F., Mora A., Fuentes J. M., Soler G., and Claro E. (1998) Partial lithium-associated protection against apoptosis induced by C2-ceramide in cerebellar granule neurons. *Neuroreport* **9**, 4199-4203.

Chen R. W. and Chuang D. M. (1999) Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. A prominent role in neuroprotection against excitotoxicity. *J. Biol. Chem.* **274**, 6039-6042.

Chen G., Huang L. D., Jiang Y. M., and Manji H. K. (1999a) The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J. Neurochem.* **72**, 1327-1330.

Chen G., Zeng W. Z., Yuan P. X., Huang L. D., Jiang Y. M., Zhao Z. H., and Manji H. K. (1999b) The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. *J. Neurochem.* **72**, 879-882.

Chen C. H., Hwang S. L., Howng S. L., Chou C. K., and Hong Y. R. (2000a) Three rat brain alternative splicing dynamin-like protein variants: interaction with the glycogen synthase kinase 3β and action as a substrate. *Biochem. Biophys. Res. Commun.* **268**, 893-898.

Chen R. H., Ding W. V., and McCormick F. (2000b) Wnt signaling to β-catenin involves two interactive components. Glycogen synthase kinase-3β inhibition and activation of protein kinase C. *J. Biol. Chem.* **275**, 17894-17899.

Chrivia J. C., Kwok R. P., Lamb N., Hagiwara M., Montminy M. R., and Goodmand R. H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859.

Chu B., Soncin F., Price B. D., Stevenson M. A., and Calderwood S. K. (1996) Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J. Biol. Chem.* **271**, 30847-30857.

Chu B., Zhong R., Soncin F., Stevenson M. A., and Calderwood S. K. (1998) Transcriptional activity of heat shock factor 1 at 37 degrees C is repressed through phosphorylation on two distinct serine residues by glycogen synthase kinase 3 and protein kinases Cα and Cζ. *J. Biol. Chem.* **273**, 18640-18646.

Coghlan M. P., Culbert A. A., Cross D. A., Corcoran S. L., Yates J. W., Pearce N. J., Rausch O. L., Murphy G. J., Carter P. S., Cox L. R., Mills D., Brown M. J., Haigh D., Ward R. W., Smith D. G., Murray K. J., Reith A. D., and Holder J. C. (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol.* **7**, 793-803.

Cook D., Fry M. J., Hughes K., Sumathipala R., Woodgett J. R., and Dale T.C. (1996) Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J.* **15**, 4526-4536.

Cross D. A., Alessi D. R., Cohen P., Andjelkovich M., and Hemmings B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.

Crowder R. J. and Freeman R. S. (2000) Glycogen synthase kinase-3β activity is critical for neuronal death caused by inhibiting PI 3-kinase or Akt but not for death caused by NGF withdrawal. *J. Biol. Chem.* **275**, 34266-34271.

Cui H., Meng Y., and Bulleit R. F. (1998) Inhibition of glycogen synthase kinase 3β activity regulates proliferation of cultured cerebellar granule cells. *Dev. Brain Res.* **111**, 177-188.

Czech C., Tremp G., and Pradier L. (2000) Presenilins and Alzheimer's disease: biological functions and pathogenic mechanisms. *Prog. Neurobiol.* **60**, 363-384.

Dale T. C. (1998) Signal transduction by the Wnt family of ligands. *Biochem. J.* **329**, 209-223.

Dang D. V., Resar L. M. S., Emison E., Kim S., Li Q., Prescott J. E., Wonsey D., and Zeller K. (1999) Function of the c-Myc oncogenic transcription factor. *Exp. Cell. Res.* **253**, 63-67.

Datta S. R., Dudek H., Tao X., Masters S., Fu H., Gotoh Y., and Greenberg M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241.

Davis G. W., Schuster C. M., and Goodman C. S. (1996) Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron* **17**, 669-679.

de Groot R. P., Auwerx J., Bourouis M., and Sassone-Corsi P. (1993) Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the *Drosophila* kinase shaggy. *Oncogene* **7**, 841-847.

Deisseroth K., Bito H., and Tsien R. W. (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron* **16**, 89-101.

del Arco G., Martinez-Martinez S., Maldonado J. L., Ortega-Perez I., and Redondo J. (2000) A role for the p38 MAP kinase pathway in the nuclear shuttling of NFATp. *J. Biol. Chem.* **275**, 13872-13878.

del Peso L., Gonzalez-Garcia M., Page C., Herrera R., and Nunez G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278**, 687-689.

Delcommenne M., Tan C., Gray V., Rue L., Woodgett J., and Dedhar S. (1998) Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc. Natl. Acad. Sci. USA* **95**, 11211-11216.

Deng T. and Karin M. (1994) c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. *Nature* **371**, 171-175.

Dent P., Campbell D. G., Hubbard M. J., and Cohen P. (1989) Multisite phosphorylation of the glycogen-binding subunit of protein phosphatase-1G by cyclic AMP-dependent protein kinase and glycogen synthase kinase-3. *FEBS Lett.* **248**, 67-72.

DePaoli-Roach A. A. (1984) Synergistic phosphorylation and activation of ATP-Mg-dependent phosphoprotein phosphatase by FA/GSK-3 and casein kinase II. *J. Biol. Chem.* **259**, 12144-12152.

DiDonato J. A., Hayakawa M., Rothwarf D. M., Zandi E., and Karin M. (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature* **388**, 548-554.

Diehl J. A., Cheng M., Roussel M. F., and Sherr C. J. (1998) Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12**, 3499-3511.

Ding X. Z., Smallridge R. C., Galloway R. J., and Kiang J. G. (1996) Increases in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and [Ca2+]i. *J. Invest. Med.* **44**, 144-153.

Ding X. Z., Tsokos G. C., and Kiang J. G. (1997) Heat shock factor-1 protein in heat shock factor-1 gene-transfected human epidermoid A431 cells requires phosphorylation before inducing heat shock protein-70 production. *J. Clin. Invest.* **99**, 136-143.

Ding V. W., Chen R. H., and McCormick F. (2000) Differential regulation of glycogen synthase kinase 3β by insulin and wnt signaling. *J. Biol. Chem.* **275**, 32475-32481.

Dragunow M., Robertson G. S., Faull R. L., Robertson H. A., and Jansen K. (1990) D2 dopamine receptor antagonists induce Fos and related proteins in rat striatal neurons. *Neuroscience* **37**, 287-294.

Duman R. S., Heninger G. R., and Nestler E. J. (1997) A molecular and cellular theory of depression. *Arch. Gen. Psychiatry* **54**, 597-606.

D'Mello S. R., Anelli R., and Calissano P. (1994) Lithium induces apoptosis in immature cerebellar granule cells but promotes survival of mature neurons. *Exp. Cell Res.* **211**, 332-338.

Eldar-Finkelman H., Seger R., Vandenheede J. R., and Krebs E. G. (1995) Inactivation of glycogen synthase kinase-3 by epidermal growth factor is mediated by mitogen-activated protein kinase/p90 ribosomal protein S6 kinase signaling pathway in NIH/3T3 cells. *J. Biol. Chem.* **270**, 987-990.

Eldar-Finkelman H. and Krebs E. G. (1997) Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc. Natl. Acad. Sci. USA* **94**, 9660-9664.

Embi N., Rylatt D. B., and Cohen P. (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur. J. Biochem.* **107**, 519-527.

Fabian J. R., Kimball S. R., Heinzinger N. K., and Jefferson L. S. (1997) Subunit assembly and guanine nucleotide exchange activity of eukaryotic initiation factor eIF2B that mediates inhibition by phosphorylated eIF2. *Mol. Cell. Biol.* **16**, 6603-6616.

Fang X., Yu S. X., Lu Y., Bast R. C. Jr., Woodgett J. R., and Mills G. B. (2000) Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc. Natl. Acad. Sci. USA* **97**, 11960-11965.

Farr G. H. 3rd, Ferkey D. M., Yost C., Pierce S. B., Weaver C., and Kimelman D. (2000) Interaction among GSK-3, GBP, axin, and APC in Xenopus axis specification. *J. Cell Biol.* **148**, 691-702.

Ferreri K., Gill G., and Montminy M. (1994) The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. *Proc. Natl. Acad. Sci. USA* **91**, 1210-1213.

Finkbeiner S. (2000) CREB couples neurotrophin signals to survival messages. *Neuron* **25**, 11-14.

Fiol C. J., Haseman J. H., Wang Y. H., Roach P. J., Roeske R. W., Kowalczuk M., and DePaoli-Roach A. A. (1988) Phosphoserine as a recognition determinant for glycogen synthase kinase-3: phosphorylation of a synthetic peptide based on the G-component of protein phosphatase-1. *Arch. Biochem. Biophys.* **267**, 797-802.

Fiol C. J., Williams, J. S., Chou C. H., Wang Q. M., Roach P. J., and Andrisani O. M. (1994) A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. *J. Biol. Chem.* **269**, 32187-32193.

Ganchi P. A., Sun S. C., Greene W. C., and Ballard D. W. (1992) IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and requires the transactivation domain to inhibit NF-κB p65 DNA binding. *Mol. Biol. Cell* **3**, 1339-1352.

Gantier R., Gilbert D., Dumanchin C., Campion D., Davoust D., Toma F. and Frebourg T. (2000) The pathogenic L392V mutation of presenilin 1 decreases the affinity to glycogen synthase kinase-3β. *Neurosci. Lett.* **283**, 217-220.

Garcia-Perez J., Avila J., and Diaz-Nido J. (1998) Implication of cyclin-dependent kinases and glycogen synthase kinase 3 in the phosphorylation of microtubule-associated protein 1B in developing neuronal cells. *J. Neurosci. Res.* **52**, 445-452.

Gibson G. E., Jope R., and Blass J. P. (1975) Decreased synthesis of acetylcholine accompanying impaired oxidation of pyruvic acid in rat brain minces. *Biochem. J.* **148**, 17-23.

Ginty D. D., Bonni A., and Greenberg M. E. (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell* **77**, 713-725.

Giordano, R. and Avantaggiati M. L. (1999) p300 and CBP: partners for life and death. *J. Cell Physiol.* **181**, 218-230.

Gonzalez G. A. and Montminy M. R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675-680.

Gonzalez G. A., Yamamoto K. K., Fischer W. H., Karr D., Menzel P., Biggs W., Vale W. W., and Montminy M. R. (1989) A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* **337**, 749-752.

Goode N., Hughes K., Woodgett J. R., and Parker P.J. (1992) Differential regulation of glycogen synthase kinase-3β by protein kinase C isotypes. *J. Biol. Chem.* **267**, 16878-16882.

Goold R. G., Owen R., and Gordon-Weeks P. R. (1999) Glycogen synthase kinase 3β phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. *J. Cell Sci.* **112**, 3373-3384.

Graef I. A., Mermelstein P. G., Stankunas K., Neilson J. R., Deisseroth K., Tsien R. W., and Crabtree G. R. (1999) L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* **401**, 703-708.

Grignon S., Levy N., Couraud F., and Bruguerolle B. (1996) Tyrosine kinase inhibitors and cycloheximide inhibit Li+ protection of cerebellar granule neurons switched to non-depolarizing medium. *Eur. J. Pharmacol.* **315**, 111-114.

Grilli M. and Memo M. (1999) Nuclear factor-κB/Rel proteins: A point of convergence of signaling pathways relevant in neuronal function and dysfunction. *Biochem. Pharmacol.* **57**, 1-7.

Grimes C. A. and Jope R.S. (2000) CREB activity is inhibited by glycogen synthase kinase-3β and facilitated by lithium. *Soc. Neurosci. Abstr.* **26**, 416.

Guan R. J., Khatra B. S., and Cohlberg J. A. (1991) Phosphorylation of bovine neurofilament proteins by protein kinase FA (glycogen synthase kinase 3). *J. Biol. Chem.* **266**, 8262-8267.

Guidato S., Tsai L. H., Woodgett J., and Miller C. C. (1996) Differential cellular phosphorylation of neurofilament heavy side-arms by glycogen synthase kinase-3 and cyclin-dependent kinase-5. *J. Neurochem.* **66**, 1698-1706.

Guo Q., Sopher B. L., Furukawa K., Pham D. G., Robinson N., Martin G. M., and Mattson M. P. (1997) Alzheimer's presenilin mutation sensitizes neural cells to apoptosis induced by trophic factor withdrawal and amyloid β-peptide: involvement of calcium and oxyradicals. *J. Neurosci.* **17**, 4212-4222.

Guzowski J. F. and McGaugh J. L. (1997) Antisense oligodeoxynucleotide-mediated disruption of hippocampal cAMP response element binding protein levels impairs consolidation of memory for water maze training. *Proc. Natl. Acad. Sci. USA* **94**, 2693-2698.

Haass C. and De Strooper B. (1999) The presenilins in Alzheimer's disease--proteolysis holds the key. *Science* **286**, 916-919.

Halazonetis T. D. and Kandil A. N. (1991) Determination of the c-Myc DNA-binding site. *Proc. Natl. Acad. Sci. USA* **88**, 6162-6166.

Halse R., Rochford J. J., McCormack J. G., Vandenheede,J. R., Hemmings B. A., and Yeaman S. J. (1999) Control of glycogen synthesis in cultured human muscle cells. *J. Biol. Chem.* **274**, 776-780.

Hanger D. P., Hughes K., Woodgett J. R., Brion J. P., and Anderton B. H. (1992) Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neurosci. Lett.* **147**, 58-62.

Hanger D. P., Betts J. C., Loviny T. L., Blackstock W. P., and Anderton B. H. (1998) New phosphorylation sites identified in hyperphosphorylated tau (paired helical

filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry. *J. Neurochem.* **71**, 2465-2476.

Haq S., Choukroun G., Kang Z. B., Ranu H., Matsui T., Rosenzweig A., Molkentin J. D., Alessandrini A., Woodgett J., Hajjar R., Michael A., and Force T. (2000) Glycogen synthase kinase-3β is a negative regulator of cardiomyocyte hypertrophy. *J. Cell. Biol.* **151**, 117-130.

Hardy J. (1997) Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci.* **20**, 154-159.

Hart M. J., de los Santos R., Albert I. N., Rubinfeld B., and Polakis P. (1998) Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β. *Curr. Biol.* **8**, 573-581.

Hartigan J. A. and Johnson G. V. W. (1999) Transient increases in intracellular calcium result in prolonged site-selective increases in Tau phosphorylation through a glycogen synthase kinase 3β-dependent pathway. *J. Biol. Chem.* **274**, 21395-21401.

He B., Meng Y. H., and Mivechi N. F. (1998) Glycogen synthase kinase 3β and extracellular signal-regulated kinase inactivate heat shock transcription factor 1 by facilitating the disappearance of transcriptionally active granules after heat shock. *Mol. Cell Biol.* **18**, 6624-6633.

Hecht A., Vleminckx K., Stemmler M. P., van Roy F., and Kemler R. (2000) The p300/CBP acetyltransferases function as transcriptional coactivators of β-catenin in vertebrates. *EMBO J.* **19**, 1839-1950.

Hedgepeth C. M., Conrad L. J., Zhang J., Huang H. C., Lee V. M., and Klein P. S. (1997) Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. *Dev. Biol.* **185**, 82-91.

Hedgepeth C. M., Deardorff M. A., Rankin K., and Klein P. S. (1999) Regulation of glycogen synthase kinase 3β and downstream Wnt signaling by axin. *Mol. Cell. Biol.* **19**, 7147-7157.

Hemati N., Ross S. E., Erickson R. L., Groblewski G. E., and MacDougald O. A. (1997) Signaling pathways through which insulin regulates CCAAT/enhancer binding protein α (C/EBP α) phosphorylation and gene expression in 3T3-L1 adipocytes. *J. Biol. Chem.* **272**, 25913-25919.

Hemmings B. A., Aitken A., Cohen P., Rymond M., and Hofmann F. (1982a) Phosphorylation of the type-II regulatory subunit of cyclic-AMP-dependent protein kinase by glycogen synthase kinase 3 and glycogen synthase kinase 5. *Eur. J. Biochem.* **127**, 473-481.

Hemmings B. A., Resink T. J., and Cohen P. (1982b) Reconstitution of a Mg-ATP-dependent protein phosphatase and its activation through a phosphorylation mechanism. *FEBS Lett.* **150**, 319-324.

Henkel T., Machleidt T., Alkalay I., Kronke M., Ben-Neriah Y., and Baeuerle P. A. (1993) Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature* **365**, 182-185.

Henriksson M., Bakardjiev A., Klein G., and Luscher B. (1993) Phosphorylation sites mapping the N-terminal domain of c-myc modulate its transforming potential. *Oncogene* **8**, 3199-3209.

Hetman M., Cavanaugh J. E., Kimelman D., and Xia Z. (2000) Role of glycogen synthase kinase-3β in neuronal apoptosis induced by trophic withdrawal. *J. Neurosci.* **20**, 2567-2674.

Hoeflich K. P., Luo J., Rubie E. A., Tsao M.-S., Jin O., and Woodgett J. R. (2000) Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation. *Nature* **406**, 86-90.

Hoey T., Sun Y. L., Williamson K., and Xu X. (1995) Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. *Immunity* **2**, 461-472.

Holmberg C. I., Leppa S., Eriksson J. E., and Sistonen L. (1997) The phorbol ester 12-O-tetradecanoylphorbol 13-acetate enhances the heat-induced stress response. *J. Biol. Chem.* **272**, 6792-6798.

Hong M., Chen D. C., Klein P. S., and Lee V. M. (1997) Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. *J. Biol. Chem.* **272**, 25326-25332.

Hong Y. R., Chen C. H., Cheng D. S., Hwong S. L., and Chow C. C. (1998) Human dynamin-like protein interacts with the glycogen synthase kinase 3β. *Biochem. Biophys. Res. Commun.* **249**, 697-703.

Hong Y., Chena C., Chang J., Wang S., Sy W., Chou C., and Hwong S. (2000) Cloning and characterization of a novel human ninein protein that interacts with the glycogen synthase kinase 3β. *Biochim. Biophys. Acta* **1492**, 513-516.

Hoshi M., Takashima A., Noguchi K., Murayama M., Sato M., Kondo S., Saitoh Y., Ishiguro K., Hoshino T., and Imahori K. (1996) Regulation of mitochondrial pyruvate dehydrogenase activity by tau protein kinase I/glycogen synthase kinase 3β in brain. *Proc. Natl. Acad. Sci. USA* **93**, 2719-2723.

Hoshi M., Takashima A., Murayama M., Yasutake K., Yoshida N., Ishiguro K., Hoshino, T., and Imahori K. (1997) Nontoxic amyloid β peptide 1-42 suppresses acetylcholine synthesis. Possible role in cholinergic dysfunction in Alzheimer's disease. *J. Biol. Chem.* **272**, 2038-2041.

Huber O., Korn R., McLaughlin J., Ohsugi M., Herrmann B. G., and Kemler R. (1996) Nuclear localization of β-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* **59**, 3-10.

Hughes K., Ramakrishna S., Benjamin W. B., and Woodgett J. R. (1992) Identification of multifunctional ATP-citrate lyase kinase as the α-isoform of glycogen synthase kinase-3. *Biochem. J.* **288**, 309-314.

Hughes K., Nikolakaki E., Plyte S. E., Totty N. F., and Woodgett J. R. (1993) Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *EMBO J.* **12**, 803-808.

Hung J. J., Cheng T. J., Lai Y. K., and Chang M. D. (1998) Differential activation of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinases confers cadmium-induced HSP70 expression in 9L rat brain tumor cells. *J. Biol. Chem.* **273**, 31924-31931.

Hurel S. J., Rochford J. J., Borthwick A. C., Wells A. M., Vandenheede J. R., Turnbull D. M., and Yeaman S. J. (1996) Insulin action in cultured human myoblasts: contribution of different signalling pathways to regulation of glycogen synthesis. *Biochem. J.* **320**, 871-877.

Ikeda S., Kishida S., Yamamoto H., Murai H., Koyama S., and Kikuchi A. (1998) Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. *EMBO J.* **17**, 1371-1384.

Imahori K. and Uchida T. (1997) Physiology and pathology of tau protein kinases in relation to Alzheimer's disease. *J. Biochem. (Tokyo)* **121**, 179-188.

Inouye M., Yamamura H., and Nakano A. (1995) Lithium delays the radiation-induced apoptotic process in external granule cells of mouse cerebellum. *J. Radiat. Res. (Tokyo)* **36**, 203-208.

Ishiguro K., Takamatsu M., Tomizawa K., Omori A., Takahashi M., Arioka M., Uchida T., and Imahori K. (1992) Tau protein kinase I converts normal tau protein into A68-like component of paired helical filaments. *J. Biol. Chem.* **267**, 10897-108910.

Ishiguro K., Shiratsuchi A., Sato S., Omori A., Arioka M., Kobayashi S., Uchida T., and Imahori K. (1993) Glycogen synthase kinase 3β is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.* **325**, 167-172.

Itoh K., Krupnik V. E., and Sokol S. Y. (1998) Axis determination in Xenopus involves biochemical interactions of axin, glycogen synthase kinase 3 and β-catenin. *Curr. Biol.* **8**, 591-594.

Jacobs B. L., Praag H., and Gage F. H. (2000) Adult brain neurogenesis and psychiatry: a novel theory of depression. *Mol. Psychiatry* **5**, 262-269.

Jain J., McCaffrey P. G., Miner Z., Kerppola T. K., Lambert J. N., Verdine G. L., Curran T., and Rao A. (1993) The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature* **365**, 352-255.

Jain J., Burgeon E., Badalian T. M., Hogan P. G., and Rao A. (1995) A similar DNA-binding motif in NFAT family proteins and the Rel homology region. *J. Biol. Chem.* **270**, 4138-4145.

Jean D., Harbison M., McConkey D. J., Ronai Z., and Bar-Eli M. (1998) CREB and its associated proteins act as survival factors for human melanoma cells. *J. Biol. Chem.* **273**, 24884-24890.

Ji L., Mochon E., Arcinas M., and Boxer L. M. (1996) CREB proteins function as positive regulators of the translocated bcl-2 allele in t(14;18) lymphomas. *J. Biol. Chem.* **271**, 22687-22691.

Johnson G. V. W. and Jope R. S. (1992) The role of microtubule-associated protein 2 (MAP-2) in neuronal growth, plasticity, and degeneration. *J. Neurosci. Res.* **33**, 505-512.

Johnson G. V. W. and Jenkins S. M. (1996) Tau protein in normal and Alzheimer's disease brain. *Alzheimer's Dis. Rev.* **1**, 38-54.

Johnson G. V. W. and Hartigan J. A. (1998) Tau protein in normal and Alzheimer's disease brain: an update. *Alzheimer's Dis. Rev.* **3**, 125-141.

Jonkers J., Korswagen H. C., Acton D., Breuer M., and Berns A. (1997) Activation of a novel proto-oncogene, Fratl, contributes to progression of mouse T-cell lymphomas. *EMBO. J.* **16**, 441-450.

Jope R. S. (1999a) Anti-bipolar therapy: Mechanism of action of lithium. *Mol. Psychiatry* **4**, 117-128.

Jope R. S. (1999b) A bimodal model of the mechanism of action of lithium. *Mol. Psychiatry* **4**, 21-25.

Jope R. S. and Song L. (1997) AP-1 and NF-κB stimulated by carbachol in human neuroblastoma SH-SY5Y cells are differentially sensitive to inhibition by lithium. *Mol. Brain Res.* **50**, 171-180.

Kaang B. K., Kandel E. R., and Grant S. G. (1993) Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in Aplysia sensory neurons. *Neuron* **10**, 427-435.

Kadoya T., Kishida S., Fukui A., Hinoi T., Michiue T., Asashima M., and Kikuchi A. (2000) Inhibition of wnt signaling pathway by a novel axin-binding protein. *J. Biol. Chem.* **275**, 37030-37037.

Kang D. E., Soriano S., Frosch M. P., Collins T., Naruse S., Sisodia S. S., Leibowitz G., Levine F., and Koo E. H. (1999) Presenilin 1 facilitates the constitutive turnover of β-catenin: differential activity of Alzheimer's disease-linked PS1 mutants in the β-catenin-signaling pathway. *J. Neurosci.* **19**, 4229-4237.

Karin M., Liu Z. G., and Zandi E. (1997) AP-1 function and regulation. *Curr. Opin. Cell Biol.* **9**, 240-246.

Khodorov B., Pinelis V., Vinskaya N., Sorokina E., Grigortsevich N., and Storozhevykh T. (1999) Li+ protects nerve cells against destabilization of Ca2+ homeostasis and delayed death caused by removal of external Na+. *FEBS Lett.* **448**, 173-176.

Kiang J. G. and Tsokos G. C. (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol. Ther.* **80**, 183-201.

Kim L., Liu J., and Kimmel A. R. (1999) The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification. *Cell* **99**, 399-408.

Kishida S., Yamamoto H., Ikeda S., Kishida M., Sakamoto I., Koyama S., and Kikuchi A. (1998) Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of β-catenin. *J. Biol. Chem.* **273**, 10823-10826.

Kleijn M., Welsh G. I., Scheper G. C., Voorma H. O., Proud C. G., and Thomas A. A. M. (1998) Nerve and epidermal growth factor induce protein synthesis and eIF2B activation in PC12 cells. *J. Biol. Chem.* **273**, 5536-5541.

Kleijn M. and Proud C. (2000) The activation of eukaryotic initiation factor (eIF)2B by growth factors in PC12 cells requires MEK/ERK signaling. *FEBS Lett.* **476**, 262-265.

Klein P. S. and Melton D. A. (1996) A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93**, 8455-8459.

Kline M. P. and Morimoto R. I. (1997) Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Mol. Cell Biol.* **17**, 2107-2115.

Konishi H., Matsuzaki H., Tanaka M., Ono Y., Tokunaga C., Kuroda S., and Kikkawa U. (1996) Activation of RAC-protein kinase by heat shock and hyperosmolarity stress through a pathway independent of phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **93**, 7639-7643.

Korinek V., Barker N., Morin P. J., van Wichen D., de Weger R., Kinzler K. W., Vogelstein B., and Clevers H. (1997) Constitutive transcriptional activation by a β-catenin-Tcf complex in $APC^{-/-}$ colon carcinoma. *Science* **275**, 1784-1787.

Kovacs D. M., Mancini R., Henderson J., Na S. J., Schmidt S. D., Kim T. W., and Tanzi R. E. (1999) Staurosporine-induced activation of caspase-3 is potentiated by presenilin 1 familial Alzheimer's disease mutations in human neuroglioma cells. *J. Neurochem.* **73**, 2278-2285.

Kovlovsky N., Belmaker R. H., and Agam G. (2000) Low GSK-3β immunoreactivity in postmortem frontal cortex of schizophrenic patients. *Amer. J. Psychiatry* **157**, 831-833.

Krylova O., Messenger M. J., and Salinas P. C. (2000) Dishevelled-1 regulates microtubule stability. A new function mediated by glycogen synthase kinase-3β. *J. Cell Biol.* **151**, 83-94.

Kucharz E. J., Sierakowski S., Staite N. D., and Goodwin J. S. (1988) Mechanism of lithium-induced augmentation of T-cell proliferation. *Int. J. Immunopharmacol.* **10**, 253-259.

Kuo C. F., Xanthopoulos K. G., and Darnell J. E. Jr. (1990) Fetal and adult localization of C/EBP: evidence for combinatorial action of transcription factors in cell-specific gene expression. *Development* **109**, 473-481.

Kwok R. P., Lundblad J. R., Chrivia J. C., Richards J. P., Bachinger H. P., Brennan R. G., Roberts S. G., Green M. R., and Goodman R. H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370**, 223-226.

Leclerc S., Garnier M., Hoessel R., Marko D., Bibb J. A., Snyder G. L., Greengard P., Biernat J., Wu Y. Z., Mandelkow E. M., Eisenbrand G., and Meijer L. (2001) Indirubins inhibit glycogen synthase kinase-3β and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease-a property common to most CDK inhibitors ? *J. Biol. Chem.* **276**, 251-260.

Lee Y. N., Park Y. G., Choi Y. H., Cho Y. S., and Cho-Chung Y. S. (2000) CRE-transcription factor decoy oligonucleotide inhibition of MCF-7 breast cancer cells: cross-talk with p53 signaling pathway. *Biochemistry* **39**, 4863-4868.

Lekstrom-Himes J. and Xanthopoulos K. G. (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.* **273**, 28545-28548.

Leost M., Schultz C., Link A., Wu Y. Z., Biernat J., Mandelkow E. M., Bibb J. A., Snyder G. L., Greengard P., Zaharevitz D. W., Gussio R., Senderowicz A. M., Sausville E. A., Kunick C., and Meijer L. (2000) Paullones are potent inhibitors of glycogen synthase kinase-3β and cyclin-dependent kinase 5/p25. *Eur. J. Biochem.* **267**, 5983-5994.

Leroy K. and Brion J.-P. (1999) Developmental expression and localization of glycogen synthase kinase-3β in rat brain. *J. Chem. Neuroanat.* **16**, 279-293.

Lesort M., Greendorfer A., Stockmeier C., Johnson G. V. W., and Jope R. S. (1999a) Glycogen synthase kinase-3β, β-catenin, and tau in postmortem bipolar brain. *J. Neural Transm.* **106**, 1217-1222.

Lesort M., Jope R. S., and Johnson G. V. W. (1999b) Insulin biphasically modulates tau phosphorylation: involvement of glycogen synthase kinase-3β and fyn tyrosine kinase. *J. Neurochem.* **72**, 576-584.

Lezoualc'h F. and Behl C. (1998) Transcription factor NF-κB: friend or foe of neurons? *Mol. Psychiatry* **3**, 15-20.

Li R., Shen Y., and El-Mallakh R. S. (1994) Lithium protects against ouabain-induced cell death. *Lithium* **5**, 211-216.

Li L., Yuan H., Weaver C. D., Mao J., Farr G. H. 3rd, Sussman D. J., Jonkers J., Kimelman D., and Wu D. (1999) Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. *EMBO J.* **18**, 4233-4240.

Li R. and El-Mallakh R. S. (2000) A novel evidence of different mechanisms of lithium and valproate neuroprotective action on human SY5Y neuroblastoma cells: caspase-3 dependency. *Neurosci. Lett.* **294**, 147-150.

Lin R. Z., Hu Z. W., Chin J. H, and Hoffman B. B. (1997) Heat shock activates c-Src tyrosine kinases and phosphatidylinositol 3-kinase in NIH3T3 fibroblasts. *J. Biol. Chem.* **272**, 31196-31202.

Loh C., Shaw K. T., Carew J., Viola J. P., Luo C., Perrino B. A., and Rao A. (1996) Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. *J. Biol. Chem.* **271**, 10884-10891.

Lovestone S., Reynolds C. H., Latimer D., Davis D. R., Anderton B. H., Gallo J. M., Hanger D., Mulot S., Marquardt B., Stabel S., Woodgett J. R., and Miller C. C. J. (1994) Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. *Curr. Biol.* **4**, 1077-1086.

Lovestone S., Davis D. R., Webster M. T., Kaech S., Brion J. P., Matus A., and Anderton B. H. (1999) Lithium reduces tau phosphorylation: effects in living cells and in neurons at therapeutic concentrations. *Biol. Psychiatry* **45**, 995-1003.

Lu R., Song L., and Jope R. S. (1999) Lithium attenuates p53 levels in human neuroblastoma SH-SY5Y cells. *Neuroreport* **10**, 1123-1125.

Lucas F. R., Goold R. G., Gordon-Weeks P. R., and Salinas P. C. (1998) Inhibition of GSK-3β leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. *J. Cell Sci.* **111**, 1351-1361.

Luo C., Burgeon E., and Rao A. (1996a) Mechanisms of transactivation by nuclear factor of activated T cells-1. *J. Exp. Med.* **184**, 141-147.

Luo C., Burgeon E., Carew J. A., McCaffrey P. G., Badalian T. M., Lane W. S., Hogan P. G., and Rao A. (1996b) Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. *Mol. Cell. Biol.* **16**, 3955-3966.

MacDougald O. A., Cornelius P., Liu R., and Lane M. D. (1995) Insulin regulates transcription of the CCAAT/enhancer binding protein (C/EBP)α, β, and δ genes in fully-differentiated 3T3-L1 adipocytes. *J. Biol. Chem.* **270**, 647-654.

Mackie K., Sorkin B. C., Nairn A. C., Greengard P., Edelman G. M., and Cunningham B. A. (1989) Identification of two protein kinases that phosphorylate the neural cell-adhesion molecule, N-CAM. *J. Neurosci.* **9**, 1883-1896.

Maggirwar S. B., Tong N., Ramirez S., Gelbard H. A., and Dewhurst S. (1999) HIV-1 Tat-mediated activation of glycogen synthase kinase-3β contributes to Tat-mediated neurotoxicity. *J. Neurochem.* **73**, 578-586.

Mandelkow E. M., Drewes G., Biernat J., Gustke N., Van Lint J., Vandenheede J. R., and Mandelkow E. (1992) Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett.* **314**, 315-321.

Mattila P. S., Ullman K. S., Fiering S., Emmel E. A., McCutcheon M., Crabtree G. R., and Herzenberg L. A. (1990) The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes. *EMBO J.* **9**, 4425-4433.

Mattson M. P. (1997) Neuroprotective signal transduction: relevance to stroke. *Neurosci. Biobehav. Rev.* **21**, 193-206.

McCaffrey P. G., Luo C., Kerppola T. K., Jain J., Badalian T. M., Ho A. M., Burgeon E., Lane W. S., Lambert J. N., and Curran T. (1993) Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. *Science* **262**, 750-754.

McMahon A. P. and Bradley A. (1990) The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

Meichle A., Philipp A., and Eilers M. (1992) The functions of Myc proteins. *Biochim. Biophys. Acta* **1114**, 129-146.

Mercurio F., Zhu H., Murray B. W., Shevchenko A., Bennett B. L., Li J., Young D. B., Barbosa M., Mann M., Manning A., and Rao A. (1997) IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. *Science* **278**, 860-866.

Mivechi N. F. and Giaccia A. J. (1995) Mitogen-activated protein kinase acts as a negative regulator of the heat shock response in NIH3T3 cells. *Cancer Res.* **55**, 5512-5519.

Molenaar M., van de Wetering M., Oosterwegel M., Peterson-Maduro J., Godsave S., Korinek V., Roose J., Destree O., and Clevers H. (1996) XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86**, 391-399.

Montminy M. R. and Bilezikjian L. M. (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328**, 175-178.

Mora A., Gonzalez-Polo R. A., Fuentes J. M., Soler G., and Centeno F. (1999) Different mechanisms of protection against apoptosis by valproate and Li+. *Eur. J. Biochem.* **266**, 886-891.

Morgan J. I., Cohen D. R., Hempstead J. L., and Curran T. (1987) Mapping patterns of c-fos expression in the central nervous system after seizure. *Science* **237**, 192-197.

Morimoto R. I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* **12**, 3788-3796.

Morin P. J., Sparks A. B., Korinek V., Barker N., Clevers H., Vogelstein B., and Kinzler K.W. (1997) Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* **275**, 1787-1790.

Morisco C., Zebrowski D., Condorelli G., Tsichlis P., Vatner S. F., and Sadoshima J., (2000) The Akt-glycogen synthase kinase 3β pathway regulates transcription of atrial natriuretic factor induced by β-adrenergic receptor stimulation in cardiac myocytes. *J. Biol. Chem.* **275**, 14466-14475.

Moule S. K., Welsh G. I., Edgell N. J., Foulstone E. J., Proud C. G., and Denton R. M. (1997) Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and β-adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms. *J. Biol. Chem.* **272**, 7713-7719.

Munoz-Montano J. R., Moreno F. J., Avila J., and Diaz-Nido J. (1997) Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons. *FEBS Lett.* **411**, 183-188.

Murai H., Okazaki M., and Kikuchi A. (1996) Tyrosine dephosphorylation of glycogen synthase kinase-3 is involved in its extracellular signal-dependent inactivation. *FEBS Lett.* **392**, 153-160.

Nagafuchi A. and Takeichi M. (1989) Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. *Cell Regul.* **1**, 37-44.

Nakamura T., Hamada F., Ishidate T., Anai K., Kawahara K., Toyoshima K., and Akiyama T. (1998) Axin, an inhibitor of the Wnt signalling pathway, interacts with β-catenin, GSK-3β and APC and reduces the β-catenin level. *Genes Cells* **3**, 395-403.

Nelson R W. and Gumbiner B. M. (1999) A cell-free assay system for β-catenin signaling that recapitulates direct inductive events in the early *Xenopus laevis* embryo. *J. Cell Biol.* **147**, 367-374.

Nikolakaki E., Coffer P. J., Hemelsoet R., Woodgett J. R., and Defize L. H. (1993) Glycogen synthase kinase 3 phosphorylates Jun family members in vitro and negatively regulates their transactivating potential in intact cells. *Oncogene* **8**, 833-840.

Noguchi K., Kitanaka C., Yamana H., Kokubu A., Mochizuki T., and Kuchino Y. (1999) Regulation of c-Myc through phosphorylation at ser-62 and ser-71 by c-Jun N-terminal kinase. *J. Biol. Chem.* **274**, 32580-32587.

Nonaka S., Hough C. J., and Chuang D. M. (1998a) Chronic lithium treatment robustly protects neurons in the central nervous system against excitotoxicity by inhibiting

N-methyl-D-aspartate receptor-mediated calcium influx. *Proc. Natl. Acad. Sci. USA* **95**, 2642-2647.

Nonaka S., Katsube N., and Chuang D. M. (1998b) Lithium protects rat cerebellar granule cells against apoptosis induced by anticonvulsants, phenytoin and carbamazepine. *J. Pharmacol. Exp. Ther.* **286**, 539-547.

Nonaka S. and Chuang D. M. (1998) Neuroprotective effects of chronic lithium on focal cerebral ischemia in rats. *Neuroreport* **9**, 2081-2084.

Novak A. and Dedhar S. (1999) Signaling through β-catenin and Lef/Tcf. *Cell. Mol. Life Sci.* **56**, 532-537.

Ohteki T., Parsons M., Zakarian A., Jones, R. G., Nguyen L. T., Woodgett J. R., and Ohashi P. S. (2000) Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. *J. Exp. Med.* **192**, 99-104.

Orford K., Crockett C., Jensen J. P., Weissman A. M., and Byers S. W. (1997) Serine phosphorylation-regulated ubiquitination and degradation of β-catenin. *J. Biol. Chem.* **272**, 24735-24738.

Ozaki N. and Chuang D.-M. (1997) Lithium increases transcription factor binding to AP-1 and cyclic AMP-responsive element in cultured neurons and rat brain. *J. Neurochem.* **69**, 2336-2344.

Ozawa M., Baribault H., and Kemler R. (1989) The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.* **8**, 1711-1717.

Pacheco M. A. and Jope R. S. (1996) Phosphoinositide signaling in human brain. *Prog. Neurobiol.* **50**, 255-273.

Palombella V. J., Rando O. J., Goldberg A. L. and Maniatis T. (1994) The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* **78**, 773-785.

Pap M. and Cooper G. M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J. Biol. Chem.* **273**, 19929-19932.

Papoulas O., Williams N. G., and Kingston R. E. (1992) DNA binding activities of c-Myc purified from eukaryotic cells. *J. Biol. Chem.* **267**, 10470-10480.

Park B. C., Kido Y., and Accili D. (1999) Differential signaling of insulin and IGF-1 receptors to glycogen synthesis in murine hepatocytes. *Biochemistry* **38**, 7517-7523.

Parker D., Ferreri K., Nakajima T., LaMorte V. J., Evans R., Koerber S. C., Hoeger C., and Montminy M. R. (1996) Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol. Cell. Biol.* **16**, 694-703.

Pascual T. and Gonzalez J. L. (1995) A protective effect of lithium on rat behaviour altered by ibotenic acid lesions of the basal forebrain cholinergic system. *Brain Res.* **695**, 289-292.

Patapoutian A. and Reichardt L. F. (2000) Roles of Wnt proteins in neural development and maintenance. *Curr. Opin. Neurobiol.* **10**, 392-399.

Pei J. J., Tanaka T., Tung Y. C., Braak E., Iqbal K., and Grundke-Iqbal I. (1997) Distribution, levels, and activity of glycogen synthase kinase-3 in the Alzheimer disease brain. *J. Neuropathol. Exp. Neurol.* **56**, 70-78.

Pei J. J., Braak E., Braak H., Grundke-Iqbal I., Iqbal K., Winblad B., and Cowburn R. F. (1999) Distribution of active glycogen synthase kinase 3β (GSK-3β) in brains staged for Alzheimer disease neurofibrillary changes. *J. Neuropathol. Exp. Neurol.* **58**, 1010-1019.

Peifer M. and Polakis P. (2000) Wnt signaling in oncogenesis and embryogenesis: a look outside the nucleus. *Science* **287**, 1606-1609.

Pennypacker K. R., Hong J.-S., and McMillian M. K. (1995) Implication of prolonged expression of Fos-related antigens. *Trends Pharmacol. Sci.* **16**, 317-321.

Plyte S. E., Hughes K., Nikolakaki E., Pulverer B. J., and Woodgett J. R. (1992) Glycogen synthase kinase-3: functions in oncogenesis and development. *Biochim. Biophys. Acta* **1114**, 147-162.

Polakis P. (2000) Wnt signaling and cancer. *Genes Dev.* **14**, 1837-1851.

Pugazhenthi S., Miller E., Sable C., Young P., Heidenreich K. A., Boxer L. M., and Reusch J. E.-B. (1999) Insulin-like growth factor-I induces bcl-2 promoter through the transcription factor cAMP-response element binding protein. *J. Biol. Chem.* **274**, 27529-27535.

Pugazhenthi S., Nesterova A., Sable C., Heidenreich K. A., Boxer L. M., Heasley L. E., and Reusch J. E. (2000) Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J. Biol. Chem.* **275**, 10761-10766.

Pulverer B. J., Kyriakis J. M., Avruch J., Nikolakaki E., and Woodgett J. R. (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature* **353**, 670-674.

Pulverer B. J., Fisher C., Vousden K., Littlewood T., Evan G., and Woodgett J. R. (1994) Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. *Oncogene* **9**, 59-70.

Quevedo C., Alcazar A., and Salinas M. (2000) Two different signal transduction pathways are implicated in the regulation of initiation factor 2B activity in insulin-like growth factor-1-stimulated neuronal cells. *J. Biol. Chem.* **275**, 19192-19197.

Ragano-Caracciolo M., Berlin W. K., Miller M. W., and Hanover J. A. (1998) Nuclear glycogen and glycogen synthase kinase 3. *Biochem. Biophys. Res. Commun.* **249**, 422-427.

Rao A., Luo C., and Hogan P. G. (1997) Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* **15**, 707-747.

Regnier C. H., Song H. Y., Gao X., Goeddel D. V., Cao Z., and Rothe M. (1997) Identification and characterization of an IκB kinase. *Cell* **90**, 373-383.

Riccio A., Ahn S., Davenport C. M., Blendy J. A., and Ginty D. D. (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science* **286**, 2358-2361.

Roach P. J. (1990) Control of glycogen synthase by hierarchal protein phosphorylation. *FASEB J.* **4**, 2961-2968.

Rogatsky I., Waase C. L., and Garabedian M. J. (1998) Phosphorylation and inhibition of rat glucocorticoid receptor transcriptional activation by glycogen synthase kinase-3 (GSK-3). Species-specific differences between human and rat glucocorticoid receptor signaling as revealed through GSK-3 phosphorylation. *J. Biol. Chem.* **273**, 14315-14321.

Ross S. E., Erickson R. L., Hemati N., and MacDougald O. A. (1999) Glycogen synthase kinase 3 is an insulin-regulated C/EBPα kinase. *Mol. Cell. Biol.* **19**, 8433-8441.

Rubinfeld B., Albert I., Porfiri R., Fiol C., Munemitsu S., and Polakis P. (1996) Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.

Rubinfeld B., Robbins P., El-Gamil M., Albert I., Porfiri E., and Polakis P. (1997) Stabilization of β-catenin by genetic defects in melanoma cell lines. *Science* **275**, 1790-1792.

Rudolph D., Tafuri A., Gass P., Hammerling G. J., Arnold B., and Schutz G. (1998) Impaired fetal T cell development and perinatal lethality in mice lacking the

cAMP response element binding protein. *Proc. Natl. Acad. Sci. USA* **95**, 4481-4486.

Ruff V. A. and Leach K. L. (1995) Direct demonstration of NFATp dephosphorylation and nuclear localization in activated HT-2 cells using a specific NFATp polyclonal antibody. *J. Biol. Chem.* **270**, 22602-22607.

Rylatt D. B., Aitken A., Bilham T., Condon G. D., Embi N., and Cohen P. (1980) Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase. *Eur. J. Biochem.* **107**, 529-537.

Saito Y., Vandenheede J. R., and Cohen P. (1994) The mechanism by which epidermal growth factor inhibits glycogen synthase kinase 3 in A431 cells. *Biochem. J.* **303**, 27-31.

Sakanaka C., Weiss J. B., and Williams L. T. (1998) Bridging of β-catenin and glycogen synthase-3β by axin and inhibition of β-catenin-mediated transcription. *Proc. Natl. Acad. Sci. USA* **95**, 3020-3023.

Saksela K., Makela T. P., Hughes K., Woodgett J. R., and Alitalo K. (1992) Activation of protein kinase C increases phosphorylation of the L-Myc trans-activator domain at a GSK-3 target site. *Oncogene* **7**, 347-353.

Sanchez C., Tompa P., Szucs K., Friedrich P., and Avila J. (1996) Phosphorylation and dephosphorylation in the proline-rich C-terminal domain of microtubule-associated protein 2. *Eur. J. Biochem.* **241**, 765-771.

Sanchez C., Diaz-Nido J., and Avila J. (2000) Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. *Prog. Neurobiol.* **61**, 133-168.

Seidensticker M. J. and Behrens J. (2000) Biochemical interactions in the wnt pathway. *Biochim. Biophys. Acta* **1495**, 168-182.

Sen R. and Baltimore D. (1986) Inducibility of kappa immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. *Cell* **47**, 921-928.

Sengupta A., Wu Q., Grundke-Iqbal I., Iqbal K., and Singh T. J. (1997) Potentiation of GSK-3-catalyzed Alzheimer-like phosphorylation of human tau by cdk5. *Mol. Cell. Biochem.* **167**, 99-105.

Seth A., Alvarez E., Gupta S., and Davis R. J. (1991) A phosphorylation site located in the NH2-domain of c-Myc increases transactivation of gene expression. *J. Biol. Chem.* **266**, 23521-23524.

Shaw J. P., Utz P. J., Durand D. B., Toole J. J., Emmel E. A., and Crabtree G. R. (1988) Identification of a putative regulator of early T cell activation genes. *Science* **241**, 202-205.

Shaw M., Cohen P., and Alessi D. R. (1997) Further evidence that the inhibition of glycogen synthase kinase-3β by IGF-1 is mediated by PDK1/PKB-induced phosphorylation of Ser-9 and not by dephosphorylation of Tyr-216. *FEBS Lett.* **416**, 307-311.

Shaw M., Cohen P., and Alessi D. R. (1998) The activation of protein kinase B by $H_2O_2$ or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. *Biochem. J.* **336**, 241-246.

Shaywitz A. J. and Greenberg M. E. (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem.* **68**, 821-861.

Sheng M., Thompson M. A., and Greenberg M. E. (1991) CREB: a $Ca^{2+}$-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427-1430.

Shibasaki R., Price E. R., Milan D., and McKeon F. (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382**, 370-373.

Silva A. J., Kogan J. H., Frankland P. W., and Kida S. (1998) CREB and memory. *Annu. Rev. Neurosci.* **21**, 127-148.

Singh L. P., Denslow N. D., and Wahba A. J. (1996) Modulation of rabbit reticulocyte guanine nucleotide exchange factor activity by casein kinases 1 and 2 and glycogen synthase kinase 3. *Biochemistry* **35**, 3206-3212.

Smeal T., Binetruy B., Mercola D. A., Birrer M., and Karin M. (1991) Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature* **354**, 494-496.

Stambolic V. and Woodgett J. R. (1994) Mitogen inactivation of glycogen synthase kinase-3β in intact cells via serine 9 phosphorylation. *Biochem. J.* **303**, 701-704.

Stambolic V., Ruel L., and Woodgett J. R. (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* **6**, 1664-1668.

Struthers R. S, Vale W. W., Arias C., Sawchenko P. E., and Montminy M. R. (1991) Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant. *Nature* **350**, 622-624.

Sutherland C., Leighton I. A., and Cohen P. (1993) Inactivation of glycogen synthase kinase-3β by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem. J.* **296**, 15-19.

Takashima A., Noguchi K., Sato K., Hoshino T., and Imahori K. (1993) Tau protein kinase I is essential for amyloid β-protein-induced neurotoxicity. *Proc. Natl. Acad. Sci. USA* **90**, 7789-7793.

Takashima A., Yamaguchi H., Noguchi K., Michel G., Ishiguro K., Sato K., Hoshino T., Hoshi M., and Imahori K. (1995) Amyloid β peptide induces cytoplasmic accumulation of amyloid protein precursor via tau protein kinase I/glycogen synthase kinase-3β in rat hippocampal neurons. *Neurosci. Lett.* **198**, 83-86.

Takashima A., Noguchi K., Michel G., Mercken M., Hoshi M., Ishiguro K., and Imahori K. (1996) Exposure of rat hippocampal neurons to amyloid β peptide (25-35) induces the inactivation of phosphatidylinositol-3 kinase and the activation of tau protein kinase I/glycogen synthase kinase-3β. *Neurosci. Lett.* **203**, 33-36.

Takashima A., Honda T., Yasutake K., Michel G., Murayama O., Murayama M., Ishiguro K., and Yamaguchi H. (1998a) Activation of tau protein kinase I/glycogen synthase kinase-3β by amyloid β peptide (25-35) enhances phosphorylation of tau in hippocampal neurons. *Neurosci. Res.* **31**, 317-323.

Takashima A., Murayama M., Murayama O., Kohno T., Honda T., Yasutake K., Nihonmatsu N., Mercken M., Yamaguchi H., Sugihara S., and Wolozin B. (1998b) Presenilin 1 associates with glycogen synthase kinase-3β and its substrate tau. *Proc. Natl. Acad. Sci. USA* **95**, 9637-9641.

Takemaru K. I. and Moon R. T. (2000) The transcriptional coactivator CBP interacts with β-catenin to activate gene expression. *J. Cell Biol.* **149**, 249-254.

Tanii H., Ankarcrona M., Flood F., Nilsberth C., Mehta N. D., Perez-Tur J., Winblad B., Benedikz E., and Cowburn R. F. (2000) Alzheimer's disease presenilin-1 exon 9 deletion and L250S mutations sensitize SH-SY5Y neuroblastoma cells to hyperosmotic stress-induced apoptosis. *Neuroscience* **95**, 593-601.

Taniuchi M., Johnson E. M. Jr., Roach P. J., and Lawrence J. C. Jr. (1986) Phosphorylation of nerve growth factor receptor proteins in sympathetic neurons and PC12 cells. In vitro phosphorylation by the cAMP-independent protein kinase FA/GSK-3. *J. Biol. Chem.* **261**, 13342-11349.

Tao X., Finkbeiner S., Arnold D. B., Shaywitz A. J., and Greenberg M. E. (1998) $Ca^{2+}$ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* **20**, 709-726.

Thomas G. M., Frame S., Goedert M., Nathke I., Polakis P., and Cohen P. (1999) A GSK3-binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of axin and β-catenin. *FEBS Lett.* **458**, 247-251.

Troussard A. A., Tan C., Yoganathan T. N., and Dedhar S. (1999) Cell-extracellular matrix interactions simulate the AP-1 transcription factor in an integrin-linked kinase- and glycogen synthase kinase 3-dependent manner. *Mol. Cell. Biol.* **19**, 7420-7427.

Tsujio I., Tanaka T., Kudo T., Nishikawa T., Shinozaki K., Grundke-Iqbal I., Iqbal K., and Takeda M. (2000) Inactivation of glycogen synthase kinase-3 by protein kinase Cδ: implications for regulation of tau phosphorylation. *FEBS Lett.* **469**, 111-117.

Urban M. B., Schreck R., and Baeuerle P. A. (1991) NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* **10**, 1817-1825.

van Weeren P. C., de Bruyn K. M., de Vries-Smits A. M., van Lint J., and Burgering B. M. (1998) Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. *J. Biol. Chem.* **273**, 13150-13156.

Volonte C. and Rukenstein A. (1993) Lithium chloride promotes short-term survival of PC12 cells after serum and NGF deprivation. *Lithium* **4**, 211-219.

Volonte C., Ciotti M. T., and Merlo D. (1994) LiCl promotes survival of GABAergic neurons from cerebellum and cerebral cortex: LiCl induces survival of GABAergic neurons. *Neurosci. Lett.* **172**, 6-10.

Waeber G. and Habener J. F. (1991) Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3',5'-monophosphate response element-binding protein CREB. *Mol. Endocrinol.* **5**, 1431-1438.

Walton M., Suara J., Young D., MacGibbon G., Hansen W., Lawlor P., Sirimanne E., Gluckman P., and Dragunow M. (1998) CCAAT-enhancer binding protein α is expressed in activated microglial cells after brain injury. *Mol. Brain Res.* **30**, 11-22.

Walton M., Woodgate A.-M., Muravlev A., Xu R., During M. J., and Dragunow M. (1999) CREB phosphorylation promotes nerve cell survival. *J. Neurochem.* **23**, 1836-1842.

Walton M. R. and Dragunow I. (2000) Is CREB a key to neuronal survival? *Trends Neurosci.* **23**, 48-53.

Waltzer L. and Bienz M. (1998) Drosophila CBP represses the transcription factor TCF to antagonize wingless signalling. *Nature* **395**, 521-325.

Wang Q. M., Fiol C. J., DePaoli-Roach A. A., and Roach P. J. (1994a) Glycogen synthase kinase-3β is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J. Biol. Chem.* **269**, 14566-14574.

Wang Q. M., Park I. K., Fiol C. J., Roach P. J., and DePaoli-Roach A. A. (1994b) Isoform differences in substrate recognition by glycogen synthase kinases 3α and 3β in the phosphorylation of phosphatase inhibitor 2. *Biochemistry* **33**, 143-147.

Wang Q. M., Roach P. J., and Fiol C. J. (1994c) Use of a synthetic peptide as a selective substrate for glycogen synthase kinase 3. *Anal. Biochem.* **220**, 397-402.

Wang J. F., Asghari V., Rockel C., and Young L. T. (1999) Cyclic AMP responsive element binding protein phosphorylation and DNA binding is decreased by chronic lithium but not valproate treatment of SH-SY5Y neuroblastoma cells. *Neuroscience* **91**, 771-776.

Webb B. and Proud C. G. (1997) Eukaryotic initiation factor 2B (eIF2B). *Int. J. Biochem. Cell. Biol.* **29**, 1127-1132.

Wei H., Leeds P. R., Qian Y., Wei W., Chen R., and Chuang D. (2000) β-amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons is inhibited by long-term lithium treatment. *Eur. J. Pharmacol.* **392**, 117-123.

Weihl C. C., Ghadge G. D., Kennedy S. G., Hay N., Miller R. J., and Roos R. P. (1999) Mutant presenilin-1 induces apoptosis and downregulates Akt/PKB. *J. Neurosci.* **19**, 5360-5369.

Welsh G. I. and Proud C. G. (1993) Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem. J.* **294**, 625-629.

Welsh G. I., Miyamoto S., Price N. T., Safer B., and Proud C. G. (1996) T-cell activation leads to rapid stimulation of translation initiation factor eIF2B and inactivation of glycogen synthase kinase-3. *J. Biol. Chem.* **271**, 11410-11413.

Welsh G. I., Stokes C. M., Wang X., Sakaue H., Ogawa W., Kasuga M., and Proud C. G. (1997) Activation of translation initiation factor eIF2B by insulin requires phosphatidyl inositol 3-kinase. *FEBS Lett.* **410**, 418-422.

Welsh G. I., Miller C. M., Loughlin A. J., Price N. T., and Proud C. G. (1998) Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett.* **421**, 125-130.

Wenzel A., Cziepluch C., Hamann U., Schurmann J., and Scwab M. (1991) The N-Myc oncoprotein is associated in vivo with the phosphoprotein Max (p20/22) in human neuroblastoma cells. *EMBO J.* **10**, 3703-3712.

Wesselborg S., Fruman D. A., Sagoo J. K., Bierer B. E., and Burakoff S. J. (1996) Identification of a physical interaction between calcineurin and nuclear factor of activated T cells (NFATp). *J. Biol. Chem.* **271**, 1274-1277.

Wilson B. E., Mochon E., and Boxer L. M. (1996) Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Mol. Cell. Biol.* **16**, 5546-5556.

Wodarz A. (1998) Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Woodgett J. R. (1990) Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J.* **9**, 2431-2438.

Woodgett J. R. (1991) cDNA cloning and properties of glycogen synthase kinase-3. *Methods Enzymol.* **200**, 564-577.

Woronicz J. D., Gao X., Cao Z., Rothe M., and Goeddel D. V. (1997) IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. *Science* **278**, 866-869.

Wu Y. Y. and Yang X. H. (1991) Enhancement of interleukin 2 production in human and Gibbon T cells after in vitro treatment with lithium. *Proc. Soc. Exp. Biol. Med.* **198**, 620-624.

Xavier I. J., Mercier P. A., McLoughlin C. L., Ali A., Woodgett J. R., and Ovsenek N. (2000) Glycogen synthase kinase 3β negatively regulates both DNA-binding and transcriptional activities of heat shock factor 1. *J. Biol. Chem.* **275**, 29147-29152.

Xia W. and Voellmy R. (1997) Hyperphosphorylation of heat shock transcription factor 1 is correlated with transcriptional competence and slow dissociation of active factor trimers. *J. Biol. Chem.* **272**, 4094-4102.

Xia W., Guo Y., Vilaboa N., Zuo J., and Voellmy R. (1998) Transcriptional activation of heat shock factor HSF1 probed by phosphopeptide analysis of factor $^{32}$P-labeled in vivo. *J. Biol. Chem.* **273**, 8749-8755.

Xie H., Litersky J. M., Hartigan J. A., Jope R. S., and Johnson G. V. W. (1998) The interrelationship between selective tau phosphorylation and microtubule association. *Brain Res.* **798**, 173-183.

Xing J., Ginty D. D., and Greenberg M.E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**, 959-963.

Yamaguchi H., Ishiguro K., Uchida T., Takashima A., Lemere C. A., and Imahori K. (1996) Preferential labeling of Alzheimer neurofibrillary tangles with antisera for tau protein kinase (TPK) I/glycogen synthase kinase-3β and cyclin-dependent kinase 5, a component of TPK II. *Acta Neuropathol. (Berlin)* **92**, 232-241.

Yamamoto H., Kishida S., Uochi T., Ikeda S., Koyama S., Asashima M., and Kikuchi A. (1998) Axil, a member of the Axin family, interacts with both glycogen synthase kinase 3β and β-catenin and inhibits axis formation of Xenopus embryos. *Mol. Cell. Biol.* **18**, 2867-2875.

Yamamoto H., Kishida S., Kishida M., Ikeda S., Takada S., and Kikuchi A. (1999) Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3β regulates its stability. *J. Biol. Chem.* **274**, 10681-10684.

Yang S. D. (1986) Identification of the ATP.Mg-dependent protein phosphatase activator (FA) as a myelin basic protein kinase in the brain. *J. Biol. Chem.* **261**, 11786-11791.

Yin J. C., Wallach J. S., Del Vecchio M., Wilder E. L., Shou H., Quinn W. G., and Tully T. (1994) Induction of a dominant negative CREB transgene specifically blocks long-term memory in Drosophila. *Cell* **79**, 49-58.

Yost C., Torres M., Miller J. R., Huang E., Kimelman D., and Moon R. T. (1996) The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443-1454.

Yost C., Farr G. H., Pierce S. B., Ferkey D. M., Chen M. M., and Kimelman D. (1998) GBP, an inhibitor of GSK-3, is implicated in Xenopus development and oncogenesis. *Cell* **93**, 1031-1041.

Yu J. S. and Yang S. D. (1994) Protein kinase FA/glycogen synthase kinase-3 predominantly phosphorylates the in vivo site Thr97-Pro in brain myelin basic protein: evidence for Thr-Pro and Ser-Arg-X-X-Ser as consensus sequence motifs. *J. Neurochem.* **62**, 1596-1603.

Yu G., Chen F., Levesque G., Nishimura M., Zhang D. M., Levesque L., Rogaeva E., Xu D., Liang Y., Duthie M., St. George-Hyslop P. H., and Fraser P. E. (1998) The

presenilin 1 protein is a component of a high molecular weight intracellular complex that contains β-catenin. *J. Biol. Chem.* **273**, 16470-16475.

Yuan P-X., Chen G., Huang L-D., and Manji H. K. (1998) Lithium stimulates gene expression through the AP-1 transcription factor pathway. *Mol. Brain Res.* **58**, 225-230.

Yukawa K., Tanaka T., Tsuji S., and Akira S. (1998) Expressions of CCAAT/enhancer-binding-proteins β and δ and their activities are intensified by cAMP signaling as well as $Ca^{2+}$/calmodulin kinase activation in hippocampal neurons. *J. Biol. Chem.* **273**, 31345-31351.

Yun Y. D., Dumoulin M., and Habener J.F. (1990) DNA-binding and dimerization domains of adenosine 3',5'-cyclic monophosphate-responsive protein CREB reside in the carboxyl-terminal 66 amino acids. *Mol. Endocrinol.* **4**, 931-939.

Zandi E., Rothwarf D. M., Delhase M., Hayakawa M., and Karin M. (1997) The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. *Cell* **91**, 243-252.

Zhou J., Liyanage U., Medina M., Ho C., Simmons A. D., Lovett M., and Kosik K. S. (1997) Presenilin 1 interaction in the brain with a novel member of the Armadillo family. *Neuroreport* **8**, 2085-2090.

# CREB DNA BINDING ACTIVITY IS INHIBITED BY GLYCOGEN SYNTHASE KINASE-3β AND IS FACILITATED BY LITHIUM

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## ABSTRACT

The regulatory influence of glycogen synthase kinase-3β (GSK3β) on the activation of cyclic AMP response element binding protein (CREB) was examined in human neuroblastoma SH-SY5Y cells. Activation of Akt with serum increased phospho-serine-9-GSK3β (the inactive form of the enzyme), inhibited GSK3β activity, and increased CREB DNA binding activity. Inhibition of GSK3β by another paradigm, treatment with the selective inhibitor lithium, also increased CREB DNA binding activity. These results indicate that inhibition of GSK3β by serine-9 phosphorylation or directly by lithium increases CREB activation, indicating that GSK3β negatively regulates CREB DNA binding activity in SH-SY5Y cells. The inhibitory regulation of CREB activation by GSK3β was also evident in differentiated SH-SY5Y cells, indicating that this regulatory interaction is maintained in nonproliferating cells. Conversely, overexpression of active GSK3β to 3.5-fold normal levels completely blocked increases in CREB DNA binding activity induced by EGF, IGF-1, forskolin, and cyclic AMP. The inhibitory effects due to overexpressed GSK3β were reversed by treatment with lithium and with another GSK3β inhibitor, sodium valproate. Overall, these results demonstrate that GSK3β inhibits, and lithium enhances, CREB activation.


## INTRODUCTION

Glycogen synthase kinase-3β (GSK3β), an enzyme first characterized by its ability to phosphorylate and inhibit glycogen synthase (Embi et al. 1980; Rylatt et al., 1980), is now recognized as a key component of several intracellular signaling systems that, among other actions, regulates the activity of multiple critical transcription factors

(Plyte et al., 1992; Kim and Kimmel, 2000). GSK3β is perhaps most well known as a component of the cell survival-promoting signaling pathway involving phosphatidylinositol 3-kinase (PI3K) and the kinase Akt (also known as protein kinase B) (Datta et al., 1999), and as an intermediate in the Wnt signaling cascade (Ferkey and Kimelman, 2000). The PI3K-Akt signaling pathway is activated by many growth factors (Datta et al., 1999), including epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1). Activated Akt phosphorylates serine-9 of GSK3β, which inhibits its kinase activity (Cross et al., 1995). Recent studies have revealed that this inhibitory control of GSK3β is an important component in the promotion of cell survival and that hyperactive GSK3β contributes to cell death (Pap and Cooper, 1998; Bijur et al., 2000; Hetman et al.. 2000). The proapoptotic action of GSK3β may be attributable, in part, to the regulation by GSK3β of the activities of an array of transcription factors, including β-catenin (Rubinfeld et al., 1996; Yost et al., 1996), AP-1 (Boyle et al., 1991), NFκB (Bournat et al., 2000), HSF-1 (Chu et al., 1996; Bijur and Jope, 2000), and others, that control the expression of numerous genes and play prominent roles in the determination of cell fate.

One of the transcription factors that may be regulated by GSK3β is the 43-kDa phosphoprotein cyclic AMP response element binding protein (CREB). CREB activity is regulated by complex phosphorylation mechanisms that are not completely characterized. Phosphorylation of CREB at serine-133 is required for recruitment of the coactivator CREB-binding protein (CBP) and transcriptional activity (Gonzalez and Montminy, 1989; Chrivia et al., 1993). Numerous signaling events can activate CREB through phosphorylation of serine-133, including activation of adenylyl cyclase, calcium

mobilization, and growth factor stimulation (Gonzalez and Montminy, 1989; Sheng et al., 1991; Ginty et al., 1994). Activation of CREB contributes to many vital processes, including cell survival (Walton and Dragunow, 2000). For example, CREB-null mice expressing functionally inactive CREB die immediately after birth (Rudolph et al., 1998), PC12 cells overexpressing CREB have decreased susceptibility to okadaic acid-induced apoptosis (Walton et al., 1996), and apoptosis is facilitated in human melanoma cells expressing dominant-negative CREB upon exposure to UV radiation (Yang et al., 1996) or thapsigargin (Jean et al., 1998). Additionally, multiple reports suggest that CREB promotes cell survival by up-regulating the expression of antiapoptotic proteins such as bcl-2 (Ji et al., 1996; Wilson et al., 1996; Pugazhenthi et al., 1999; Riccio et al., 1999). These and other findings indicate that the regulation of CREB activity is critical for cell survival and other functions (Walton and Dragunow, 2000).

Phosphorylation of CREB at serine-133 creates a consensus site for phosphorylation by GSK3β at serine-129 (Fiol et al., 1987, 1994; Wang et al., 1994; Bullock and Habener, 1998). Two studies have addressed the functional consequences of this hierarchical phosphorylation of CREB by GSK3β, but the two reached opposite conclusions. Fiol et al. (1994) reported that activation of CREB in response to cyclic AMP was potentiated in F9 cells overexpressing wild-type GSK3β and was impaired in PC12 cells expressing CREB with a mutation in the GSK3β phosphorylation site, suggesting that GSK3β facilitated activation of CREB. In contrast, Bullock and Habener (1998) found that phosphorylation of CREB by GSK3β attenuated protein kinase A-induced CREB DNA binding activity. Thus, although there is a consensus that CREB is phosphorylated by GSK3β, the functional outcome of this modification remains to be

clarified. Therefore, this study investigated the regulatory effects of GSK3β on CREB in human neuroblastoma SH-SY5Y cells. Based on several experimental paradigms, the results show that GSK3β negatively regulates CREB DNA binding activity and that lithium and sodium valproate, inhibitors of GSK3β, facilitate CREB activation.

## MATERIALS AND METHODS

### *Cell Culture*

Human neuroblastoma SH-SY5Y cells were grown on Corning 100-mm-diameter tissue culture dishes (Corning, NY, U.S.A.) in continuous culture RPMI 1640 medium (Cellgro, Herndon, VA, U.S.A.) containing 10% horse serum (Life Technologies, Gaithersburg, MD, U.S.A.), 5% fetal clone II (Hyclone, Logan, UT, U.S.A.), 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). SH-SY5Y cells were differentiated to a neuronal phenotype as described previously (Sayas et al., 1999) by maintaining cells in neurobasal medium supplemented with B-27 (Life Technologies) for 3.5 days. On the third day, serum was withdrawn, and cells were maintained in neurobasal medium without B-27 for 24 h. Stably transfected SH-SY5Y cells overexpressing HA-tagged GSK3β were described previously (Bijur et al., 2000) and were maintained in medium containing 100 μg/ml G418 (Geneticin, Alexis Biochemicals, San Diego, CA). Cells were maintained in humidified, 37°C chambers with 5% $CO_2$. For all experiments, cells were plated at a density of $10^5$ cells per 100-mm-diameter dish, serum was withdrawn after 24 h (unless stated otherwise), and cells were harvested ~24 h later, following treatments described in Results. Cyclic AMP

levels were measured with a kit according to the supplier's instructions (Amersham, Arlington Heights, IL, U.S.A.).

*Immunoblotting and Immunoprecipitation*

Cells were washed twice with phosphate-buffered saline and were lysed with 500 µl of lysis buffer [20 m*M* Tris (pH 7.5), 150 m*M* NaCl, 2 m*M* EDTA, 2 m*M* EGTA, 0.2% NP-40, 1 m*M* sodium orthovanadate, 100 µ*M* phenylmethanesulfonyl fluoride, 0.2 m*M* okadaic acid, and 10 µg/ml each of leupeptin, aprotinin, and pepstatin]. The lysates were collected in microcentrifuge tubes, sonicated for 10 s, and centrifuged at 20,800 *g* for 15 min. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, U.S.A.). The lysates were stored at -80°C until used for immunoblotting.

Cell lysates were mixed with Laemmli sample buffer [2% sodium dodecyl sulfate (SDS)] and placed in a boiling water bath for 10 min. Proteins were separated in 8% SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose. Blots were probed with antibodies to Akt, phospho-Akt, CREB, phospho-CREB (Cell Signaling Technology, Beverly, MA, U.S.A.), GSK3β (Pharmingen/Transduction Laboratories, San Diego, CA, U.S.A.), or CBP (Santa Cruz Biotechnology, CA, U.S.A.), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, U.S.A.). Blots were developed using peroxidase substrate chemiluminescence (Amersham-Pharmacia, Piscataway, NJ, U.S.A.) and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

To measure levels of phospho-serine-9-GSK3β, cell lysates were incubated with 2 μg of phospho-serine-9-GSK3β polyclonal antibody (Biosource, Camarillo, CA, U.S.A.) overnight at 4°C and incubated with 60 μl of protein A sepharose beads (Sigma, St. Louis, MO, U.S.A.) for 1 h at 4°C with gentle agitation. Immune complexes were washed and incubated in a boiling water bath, proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE), and immunoblots were obtained using a GSK3β monoclonal antibody (Pharmigen/Transduction Laboratories).

For coimmunoprecipitation experiments, cell lysates were incubated with 2 μg of CBP monoclonal antibody (Pharmingen) overnight at 4°C. Samples were incubated with 60 μl of protein G sepharose beads (Amersham-Pharmacia) for 1 h at 4°C with gentle agitation. The immune complexes were washed three times with lysis buffer without protease and phosphatase inhibitors. Samples in Laemmli buffer were placed in a boiling water bath, proteins were separated by SDS-PAGE, and samples were immunoblotted for phospho-CREB or CBP.

*Electrophoretic Mobility Shift Assay (EMSA)*

Cells were washed two times with 4 ml of phosphate-buffered saline and lysed with 500 μl of Nonidet P-40 lysis buffer [10 m*M* Tris-Cl (pH 7.4), 3 m*M* $MgCl_2$, 10 m*M* NaCl, and 0.5% Nonidet P-40]. Cell lysates were centrifuged at 4,000 *g* for 5 min at 4°C. The pellet was resuspended in 50 μl of buffer [20 mM HEPES (pH 7.9), 20% glycerol, 0.3 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 1 m*M* dithiothreitol, 0.1 m*M* β-glycerophosphate, 0.5 m*M* vanadate, 1 m*M* phenylmethanesulfonyl fluoride, and 1 μg/ml each of pepstatin A, leupeptin, and aprotinin]. After a 30-min extraction on ice, samples

were centrifuged at 16,000 *g* for 15 min at 4°C. The supernatant containing nuclear extracts was transferred to a sterile microcentrifuge tube, and protein concentrations were determined by the method of Bradford (1976).

A 17-bp double-stranded oligonucleotide containing the consensus sequence for CREB 5'-(TCG AGC TGA CGT CAG AG)-3' was used for EMSAs (Integrated DNA Technologies, Coralville, IA, U.S.A.). Double-stranded oligonucleotide (200 pmol) was radiolabeled by incubating for 1 h at 37°C in 20 μl containing reaction buffer (Amersham-Pharmacia), DNA polymerase I (Klenow Enzyme; Amersham-Pharmacia), and 100 μCi of [$\alpha^{32}$P]dCTP (Amersham, Arlington Heights, IL, U.S.A.). After incubation, samples were diluted to 50 μl with sterile TE buffer [10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA], and free probe was removed by centrifugation at 3,000 rpm for 2 min through ProbeQuant G-50 microcolumns (Amersham-Pharmacia).

DNA binding was measured by incubating nuclear extracts (10 μg of protein) in 20 μl of binding buffer containing 20 m*M* HEPES (pH 7.0), 4% glycerol, 500 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 1 mg of poly(dI-dC), and ~12,000 cpm of radiolabeled oligonucleotide for 30 min at 4°C. For supershift experiments, nuclear extracts were incubated with an antibody (0.5 μg) to CREB (Cell Signaling Technology), Fos (Calbiochem, La Jolla, CA, U.S.A.), or Egr-1 (Santa Cruz Biotechnology) for 30 min before incubation with binding buffer. Reaction mixtures were electrophoresed on 6% nondenaturing polyacrylamide gels in 0.25X TBE (22.3 m*M* Tris, 22.3 m*M* boric acid, and 0.5 m*M* EDTA) for 1.5 h at 150 V. The gels were then vacuum-dried, exposed to a phosphorscreen overnight, and quantitated using a PhosphorImager (Molecular

Dynamics, Sunnyvale, CA, U.S.A.). All experiments were carried out at least three times, and statistical significance was determined by ANOVA.

*GSK3β Activity Assay*

The activity of GSK3β was measured essentially as described previously (Lesort et al., 1999). Cells were lysed in immunoprecipitation lysis buffer [20 m*M* Tris (pH 7.5), 0.2% Nonidet P-40, 150 m*M* NaCl, 2 m*M* EDTA, 2 m*M* EGTA, 1 m*M* sodium orthovanadate, 100 μ*M* phenylmethanesulfonyl fluoride, 1 n*M* okadaic acid, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin]. The lysates were sonicated in microcentrifuge tubes for 10 s on ice and centrifuged at 20,800 *g* for 15 min. Lysates were precleared with 40 μl of protein G-Sepharose beads (Amersham-Pharamcia) for 15 min at 4°C, protein concentrations were determined, and 100 μg of protein (1 μg/μl) was incubated with 1.5 μg of monoclonal GSK3β antibody (Pharmingen/Transduction Laboratories) for 2 h at 4°C with gentle agitation. Lysates were then incubated with 60 μl of protein G-sepharose for 1 h at 4°C. The immobilized immune complexes were washed twice with immunoprecipitation lysis buffer and twice with kinase buffer [20 m*M* Tris (pH 7.5), 5 m*M* $MgCl_2$, and 1 m*M* dithiothreitol]. Kinase activity was measured by mixing immunoprecipitated GSK3β with 25 μl of kinase buffer containing 20 m*M* Tris (pH 7.5), 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 250 μ*M* ATP, 1.4 μCi of [$\gamma$-$^{32}$P]ATP (Amersham), varying concentrations of lithium (when indicated), and 0.1 μg/μl of recombinant tau protein (provided by Dr. Gail V. W. Johnson, University of Alabama at Birmingham). The samples were incubated at 30°C for 15 min, and 25 μl of Laemmli sample buffer (2% SDS) was added to each sample to stop the reaction. Samples were

placed in a boiling water bath for 10 min, and proteins were separated in 8% SDS-polyacrylamide gels. The gels were vacuum-dried, exposed to a phosphorscreen overnight, and quantitated using a PhosphorImager (Molecular Dynamics). The efficiency of GSK3β immunoprecipitation was determined by immunoblotting for GSK3β.

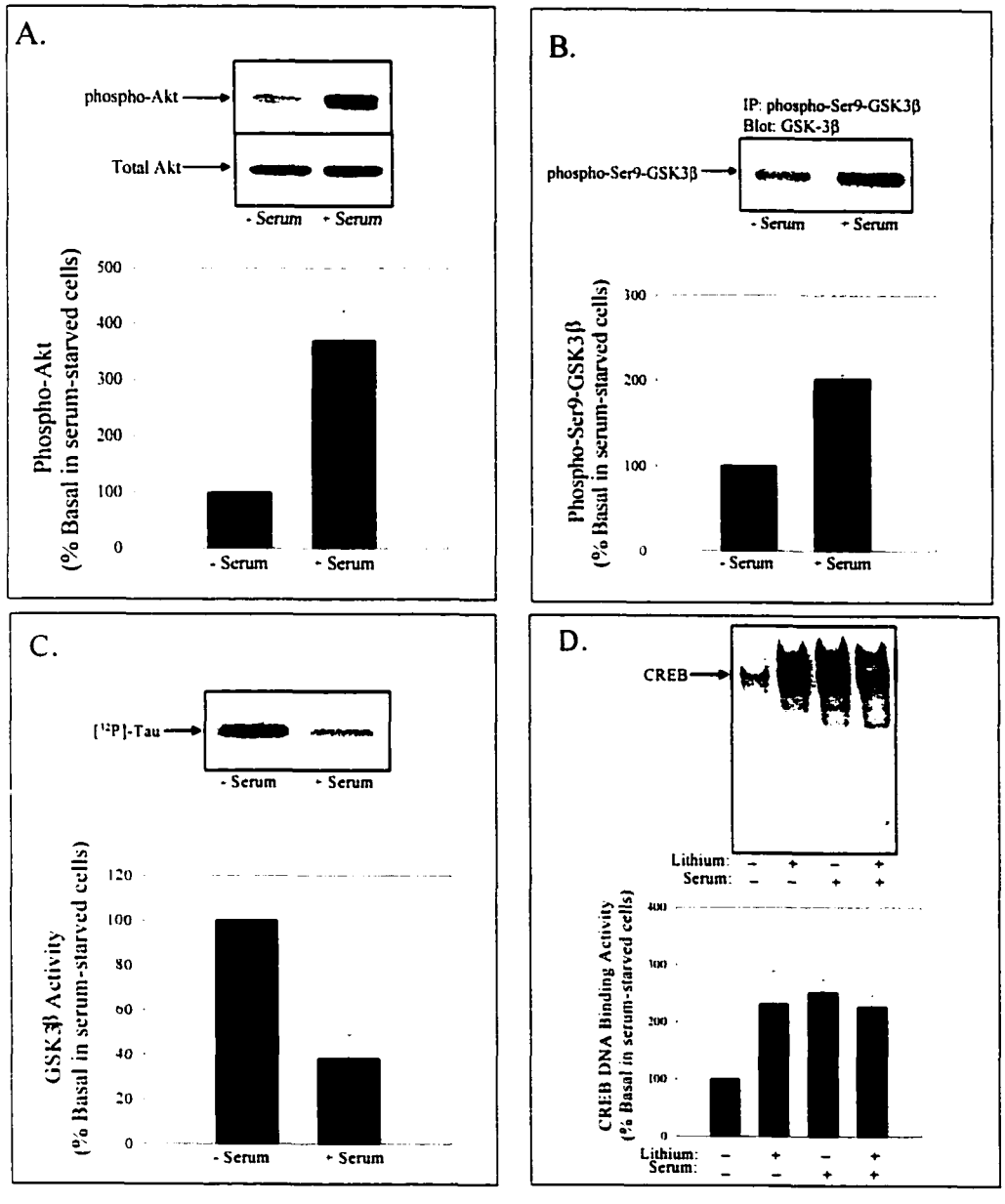
## RESULTS

*Reduction of GSK3β activity increases CREB DNA binding activity*

To determine if GSK3β has an inhibitory influence on CREB DNA binding activity, two treatment protocols were used to reduce the activity of GSK3β in wild-type SH-SY5Y cells. First, GSK3β activity was reduced indirectly by using serum to stimulate the activation of Akt, which inhibits GSK3β activity by phosphorylating serine-9 of GSK3β (Cross et al., 1995). Second, GSK3β activity was reduced directly by treating cells with lithium, a selective inhibitor of GSK3β (Klein and Melton, 1996; Stambolic et al., 1996).

The activation state of Akt was modulated by maintaining cells in growth media containing serum for comparison with results obtained in serum-withdrawn (24 h) cells. The phosphorylated, active form of Akt was 3.7-fold higher in cells maintained in medium containing serum compared with cells that were serum-starved for 24 h, although total Akt levels remained unchanged (Fig. 1A). The greater amount of active, phosphorylated Akt in cells in serum-containing medium was associated with more of the inhibited phospho-serine-9 form of GSK3β, which was 200 ± 12% of the level in serum-starved cells (Fig. 1B), and a level of GSK3β activity that was 38 ± 10% of the activity in

**FIG. 1.** Serum decreases GSK3β activity and increases CREB DNA binding activity in SH-SY5Y cells. SH-SY5Y cells were serum-starved for 24 h unless stated otherwise. **A:** Phosphorylated and total levels of Akt were measured in lysates from cells incubated without or with serum by immunoblot analysis. Quantitative values are expressed as percentages of phosphorylated Akt levels in serum-starved cells. Data are mean ± SEM (bars) values (n = 4). **B:** Phosphorylated serine-9-GSK3β levels were measured in lysates from cells incubated without or with serum by immunoprecipitating phospho-serine-9-GSK3β and immunoblotting GSK3β. Quantitative values are expressed as percentages of phosphorylated serine-9-GSK3β in serum-starved cells. Data are mean ± SEM (bars) values (n = 3). **C:** GSK3β activity was measured by immunoprecipitating GSK3β from cells incubated without or with serum, incubating the immunoprecipitated GSK3β with recombinant human tau and [$^{32}$P]ATP, and measuring the phosphorylation of tau, as described in Materials and Methods. Quantitative values are expressed as percentages of GSK3β activity in serum-starved cells. Data are mean ± SEM (bars) values (n = 4). **D:** CREB DNA binding activity was measured in nuclear extracts from cells incubated without or with serum and treated without or with lithium (1 m*M* for 1 h). Quantitative values are expressed as percentages of CREB DNA binding activity in serum-starved cells. Data are mean ± SEM (bars) values (n = 4).



serum-starved cells (Fig. 1C). In association with the decreased activity of GSK3β in cells maintained in serum, CREB DNA binding activity was 232 ± 58% of that in serum-starved cells (Fig. 1D). Thus, in cells maintained in serum, compared with serum-starved cells, Akt activity was higher, GSK3β activity was lower, and CREB DNA binding activity was higher. These results are consistent with the hypothesis that GSK3β is an inhibitory modulator of CREB DNA binding activity.

To test whether the higher GSK3β activity contributed to the lower CREB DNA binding activity in serum-withdrawn cells compared with cells maintained in serum, GSK3β was inhibited directly by treating cells with 1 m*M* lithium for 30 min. In serum-starved cells, which contained greater GSK3β activity and less of the inactive phospho-serine-9-GSK3β, this treatment with lithium to inhibit GSK3β increased CREB DNA binding activity 2-fold (Fig. 1D). In contrast, lithium treatment did not have a discernable effect on CREB DNA binding activity in serum-treated cells in which GSK3β was already inhibited by the active Akt, supporting the conclusion that lithium increased CREB DNA binding activity through its inhibitory effect on GSK3β.

Supershift analyses were used to confirm the identity of the CREB DNA binding activity obtained with the EMSA (Fig. 2). Using cells from which serum was withdrawn for 24 h, an antibody specific for CREB completely supershifted the CREB DNA binding activity to a slower mobility in nuclear extracts from both untreated cells and from cells in which CREB was activated by treatment with 1 m*M* lithium for 30 min. In contrast, Fos and Egr-1 antibodies did not affect the CREB DNA binding band intensity or mobility.

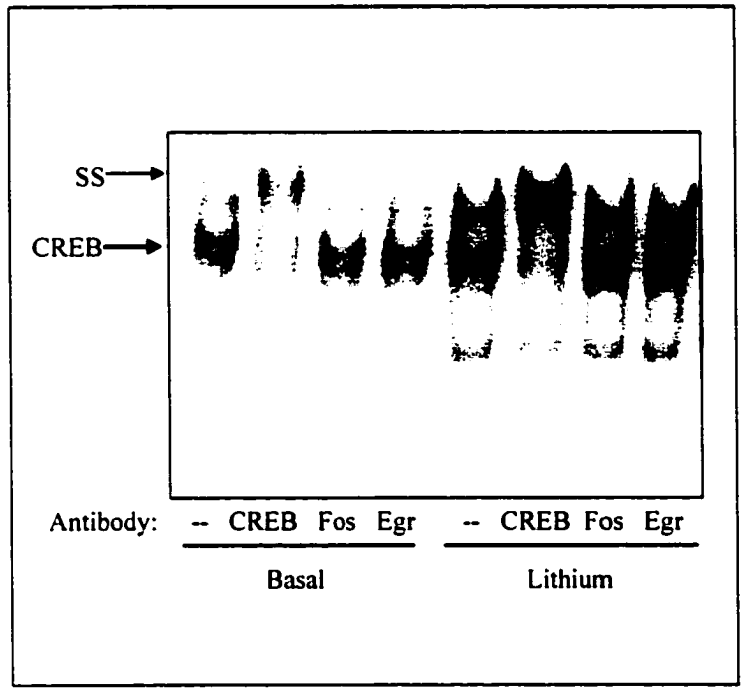



**FIG. 2.** Supershift analysis of CREB DNA binding activity in SH-SY5Y cells. CREB DNA binding activity was measured by EMSA in nuclear extracts prepared from serum-starved (24 h) SH-SY5Y cells incubated without (Basal) or with lithium (1 m*M* for 1 h). For supershift (ss) analysis, nuclear extracts were incubated with antibody (0.5 μg) to CREB, Fos, or Egr-1 before incubation with radiolabeled oligonucleotide.

To examine if the inverse relationship between GSK3β and CREB DNA binding activity was maintained in nonproliferating cells, SH-SY5Y cells were differentiated for 3.5 days, as described previously (Sayas et al., 1999), followed by serum withdrawal. Inhibition of GSK3β with lithium (1 m*M*; for 1 h) increased CREB DNA binding activity in serum-starved differentiated SH-SY5Y cells (Fig. 3) but had no effect in serum-containing cells, indicating that these responses are not limited to proliferating cells. Taken together, these results indicate that GSK3β has an inhibitory influence on CREB DNA binding activity and that inhibition of GSK3β either by activation of Akt or with lithium increased CREB DNA binding activity.

*Stimulation of phospho-CREB-CBP complex formation and CREB DNA binding activity*

To examine the modulatory effects of GSK3β in further experiments, treatments were identified that activate CREB using two assessments: the formation of complexes containing phospho-CREB and CBP, and CREB DNA binding activity. Phosphorylation of CREB at serine-133 enables complex formation with CREB-binding protein (CBP), and recruitment of CBP by CREB has been shown to be sufficient for transcriptional activation (Cardinaux et al., 2000). This complex formation was examined by measuring the amount of phospho-CREB that coimmunoprecipitated with CBP. In serum-starved wild-type SH-SY5Y cells, treatment with EGF or IGF-1 increased the amount of phospho-CREB that coimmunoprecipitated with CBP (Fig. 4A). Additionally, treatment with lithium (10 m*M* for 30 min) to inhibit GSK3β increased the coimmunoprecipitation of phospho-CREB with CBP (Fig. 4A). Measurements of CREB DNA binding activity demonstrated that treatment with EGF, IGF-1, or forskolin, an activator of adenylyl

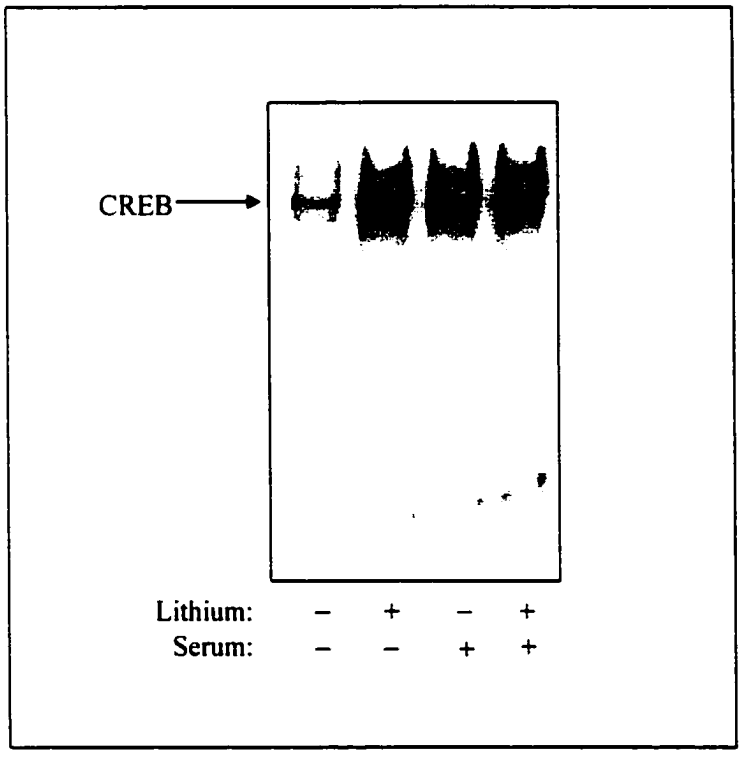



**FIG. 3.** Lithium stimulates CREB DNA binding activity following serum withdrawal in differentiated SH-SY5Y cells. SH-SY5Y cells were differentiated to a neuronal-like phenotype by maintaining cells in neurobasal medium, supplemented with B27, for 3.5 days. To withdraw serum, B27 supplement was removed 24 h before preparation of nuclear extracts. CREB DNA binding activity was measured by EMSA in nuclear extracts prepared from serum-containing or serum-starved cells treated without or with lithium (1 m*M*; 1 h).

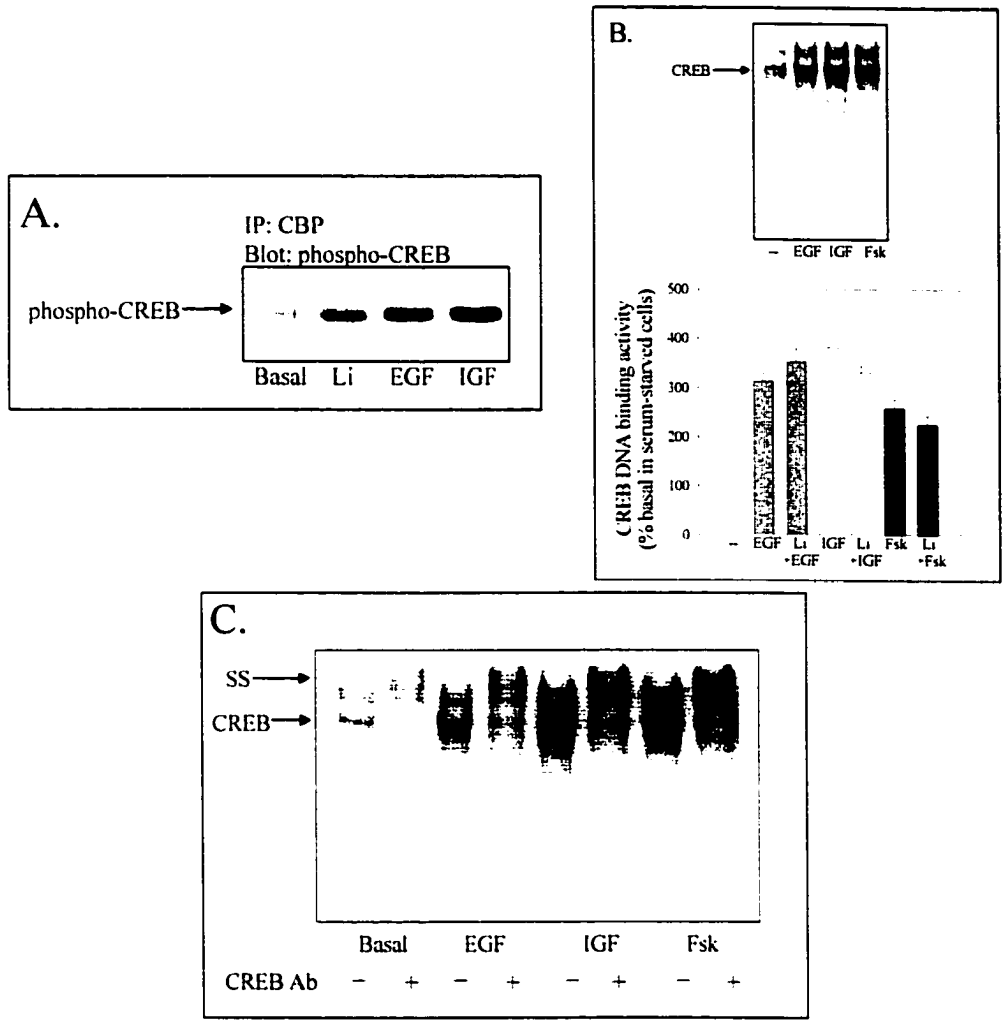



**FIG. 4.** EGF, IGF-1, and forskolin stimulate CREB DNA binding activity in serum-starved SH-SY5Y cells. **A:** The association of phospho-CREB with CBP was measured by immunoprecipitating CBP from serum-starved (24 h) wild-type SH-SY5Y cells treated without or with lithium (Li, 10 m*M* for 30 min), EGF (50 ng/ml for 30 min), or IGF-1 (50 ng/ml for 30 min) and immunoblotting phospho-CREB, as described in Materials and Methods. **B:** CREB DNA binding activity was measured by EMSA in nuclear extracts prepared from SH-SY5Y cells incubated without (24 h) and treated without (Basal) or with EGF (50 ng/ml for 1 h), IGF-1 (50 ng/ml for 1 h), or forskolin (Fsk; 10 μ*M* for 1 h) following preincubation without or with lithium (Li; 5 m*M*; 1 h). Quantitative values are expressed as percentages of basal CREB DNA binding activity in serum-starved cells. Data are mean ± SEM (bars) values (n = 4-6). **C:** Supershift (ss) analysis of CREB DNA binding activity. Nuclear extracts prepared from serum-starved (24 h) cells treated without (Basal) or with EGF (50 ng/ml for 1 h), IGF-1 (50 ng/ml for 1 h), or forskolin (Fsk; 10 μ*M* for 1 h) were incubated with antibody to CREB (0.5 μg).

cyclase, increased CREB DNA binding activity to 314 ± 15%, 381 ± 18%, and 258 ± 19%, respectively, of the basal CREB DNA binding activity (Fig. 4B). Pretreatment with lithium (1 m*M* for 1 h) did not alter activation of CREB DNA binding activity induced by any of these stimuli (Fig. 4B). Incubation with an antibody specific for CREB supershifted the CREB DNA binding activity to a slower mobility in nuclear extracts from cells treated with each of the stimulants (Fig. 4C).

*Increased GSK3β activity inhibits stimulation of CREB DNA binding activity*

To complement the experiments that showed that inhibition of GSK3β was associated with increased CREB DNA binding activity, experiments were carried out to determine whether increased GSK3β activity inhibits the activation of CREB. Since no specific activators of GSK3β are known, these experiments used stable lines of SH-SY5Y cells overexpressing GSK3β (Bijur et al., 2000). These GSK3β-transfected SH-SY5Y stable cell lines have been shown to have 3- to 4-fold higher levels (Fig. 5A) and activities (Bijur et al., 2000) of GSK3β than wild-type SH-SY5Y cells. Initial signaling induced by EGF and IGF-1, as deduced from measurements of stimulant-induced increases in Akt phosphorylation, were identical in control and GSK3β-overexpressing cells (Fig. 5B). Additionally, treatment with EGF, IGF-1, or lithium increased the coimmunoprecipitation of phospho-CREB with CBP in GSK3β-overexpressing cells (Fig. 5*C*). This is in accordance with the findings of Bullock and Habener (1998) that the inhibitory action of GSK3β should not influence the association of phospho-CREB with CBP, but should only inhibit CREB DNA binding activity. This was found to be the

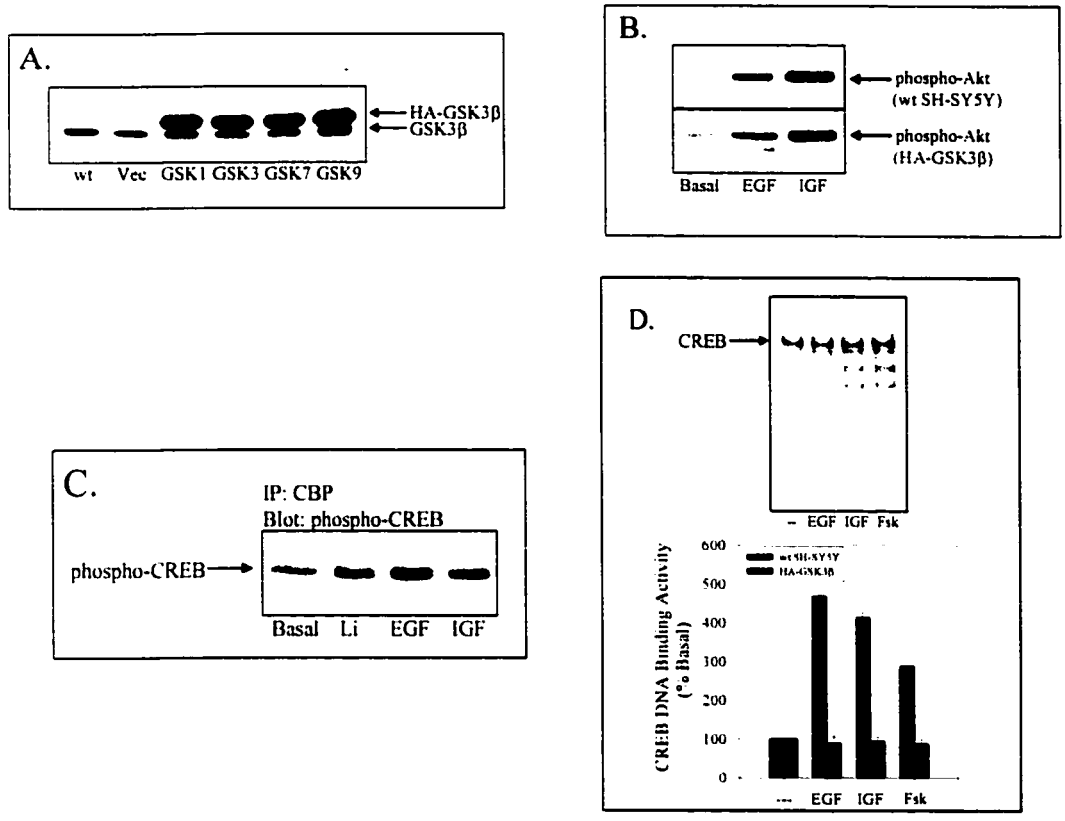



**FIG. 5.** Stimulation of CREB DNA binding activity is impaired in SH-SY5Y cells overexpressing GSK3β. **A:** Cell lysates from wild-type SH-SY5Y cells (wt), vector-transfected SH-SY5Y cells (Vec), and four cell lines stably overexpressing HA-GSK3β (1, 3, 7, and 9) were immunoblotted for GSK3β. **B:** Serum-starved (24 h) wild-type SH-SY5Y cells and GSK3β-overexpressing cells were treated without (Basal) or with EGF (50 ng/ml for 1 h) or IGF-1 (50 ng/ml for 1 h), and lysates were immunoblotted for phosphorylated Akt. **C:** The association of phospho-CREB with CBP was measured by immunoprecipitating CBP from serum-starved (24 h) GSK3β-overexpressing SH-SY5Y cells treated without or with lithium (20 m*M* for 30 min), EGF (50 ng/ml for 30 min), or IGF-1 (50 ng/ml for 30 min) and immunoblotting phospho-CREB, as described in Materials and Methods. **D:** CREB DNA binding activity was measured by EMSA in nuclear extracts prepared from wild-type and GSK3β-overexpressing cells incubated without serum (24 h) and treated without (Basal) or with EGF (50 ng/ml for 1 h), IGF-1 (50 ng/ml for 1 h), or forskolin (Fsk; 10 μ*M* for 1 h). Quantitative values are expressed as percentages of basal CREB DNA binding activity in serum-starved cells. Data are mean ± SEM (bars) values (n = 4-6).

case, as in cells overexpressing GSK3β, EGF-, IGF-1-, and forskolin-stimulated CREB DNA binding activity was almost completely blocked (Fig. 5D). To ensure that the impaired responses in cells overexpressing GSK3β were not clone-specific, three additional SH-SY5Y cell lines overexpressing GSK3β were tested. In each of these cell lines, stimulation of CREB DNA binding activity by EGF, IGF-1, or forskolin was blocked (Fig. 5D). These results further support the conclusion that GSK3β is an inhibitory modulator of CREB.To confirm that the GSK3β overexpression-induced inhibition of growth factor-stimulation of CREB was not specific to one time point of treatment, the time courses of IGF-1- and EGF-stimulated CREB DNA binding activity were measured in wild-type, vector-transfected, and GSK3β-overexpressing cells. Treatment of wild-type and vector-transfected SH-SY5Y cells with EGF or IGF-1 resulted in robust, time-dependent increases in CREB DNA binding activity, which increased 3- to 5-fold within 15 min of treatment and remained significantly elevated for at least 60 min (Fig. 6A). In contrast, in SH-SY5Y cells overexpressing GSK3β, stimulation of CREB DNA binding activity in response to EGF or IGF-1 was completely blocked throughout 60 min of treatment. To further confirm that the impaired activation of CREB DNA binding activity could be due to inadequate inhibition of GSK3β following growth factor treatments, GSK3β activity was measured in GSK3β-overexpressing SH-SY5Y cells before and after treatments with EGF or IGF-1. EGF and IGF-1 treatment reduced GSK3β activity in GSK3β-overexpressing cells, but the activity of GSK3β remained well above the activity levels in control cells (Fig. 6B). This finding is consistent with the conclusion that GSK3β_ negatively regulates CREB activity.

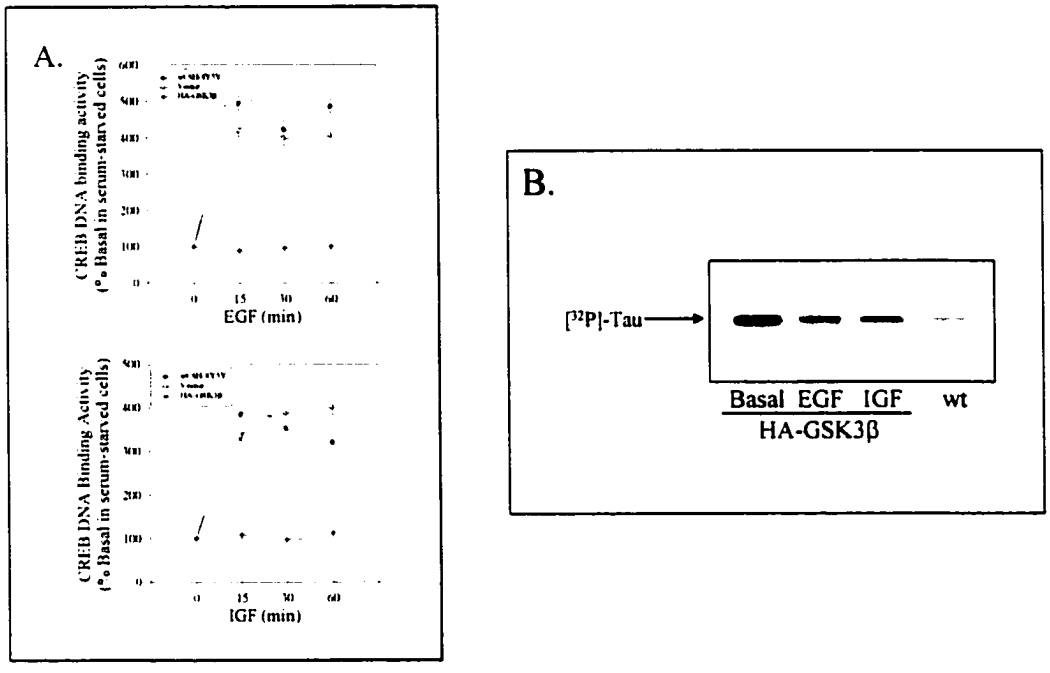



**FIG. 6.** Growth factor stimulation of CREB DNA binding activity is blocked at multiple time points in SH-SY5Y cells overexpressing GSK3β. **A:** CREB DNA binding activity was measured in nuclear extracts prepared from wild-type, vector-transfected, and HA-GSK3β-overexpressing SH-SY5Y cells incubated without serum (24 h) and treated without or with EGF (50 ng/ml) or IGF-1 (50 ng/ml). Quantitative values are expressed as percentages of basal CREB DNA binding activity in serum-starved cells. Data are mean ± SEM (bars) values (n = 3-4). **B:** In wild-type (wt) or GSK3β-overexpressing SH-SY5Y cells (GSK3β) incubated without serum (24 h) and treated without (Basal) or with EGF (50 ng/ml for 1 h) or IGF-1 (50 ng/ml for 1 h), GSK3β activity was measured by immunoprecipitating GSK3β, incubating the immunoprecipitated GSK3β with recombinant human tau and [$^{32}$P]ATP, and measuring the phosphorylation of tau, as described in Materials and Methods

*Inhibitors of GSK3β restore stimulation of CREB DBA binding activity in cells overexpressing GSK3β*

To test further if increased GSK3β activity caused the attenuation of growth factor-stimulated CREB activity in GSK3β-overexpressing cells, experiments were carried out with lithium to inhibit GSK3β. To determine the concentration of lithium necessary to reduce the GSK3β activity to below that present in wild-type SH-SY5Y cells, the lithium concentration-dependent inhibition of GSK3β activity was measured in GSK3β immunoprecipitates from GSK3β-overexpressing SH-SY5Y cells. As reported previously (Klein and Melton, 1996), lithium directly inhibited GSK3β activity in these in vitro assays (Fig. 7). The lithium concentration-dependent inhibition of GSK3β revealed that in GSK3β-overexpressing cells 20 m*M* lithium was needed to reduced the GSK3β activity to a level below that in control untreated cells. On the basis of this result, a concentration of 20 m*M* lithium was used to inhibit GSK3β activity in cells overexpressing GSK3β, although it is possible that the intracellular lithium concentration is less than 20 m*M* at the short incubation times used. Measurements of CREB DNA binding activity in cells overexpressing GSK3β revealed that stimulation by EGF and IGF-1 was restored by pretreatment with lithium (Fig. 8A and B). These findings indicate that for EGF and IGF-1 to activate CREB DNA binding activity, inhibition of GSK3β activity is a necessary component of the signaling cascade. In contrast to the results obtained with EGF and IGF-1, lithium had no effect on the impaired forskolin-induced stimulation of CREB DNA binding activity in cells overexpressing GSK3β (data not shown), indicating that another mechanism accounted for deficient CREB activation

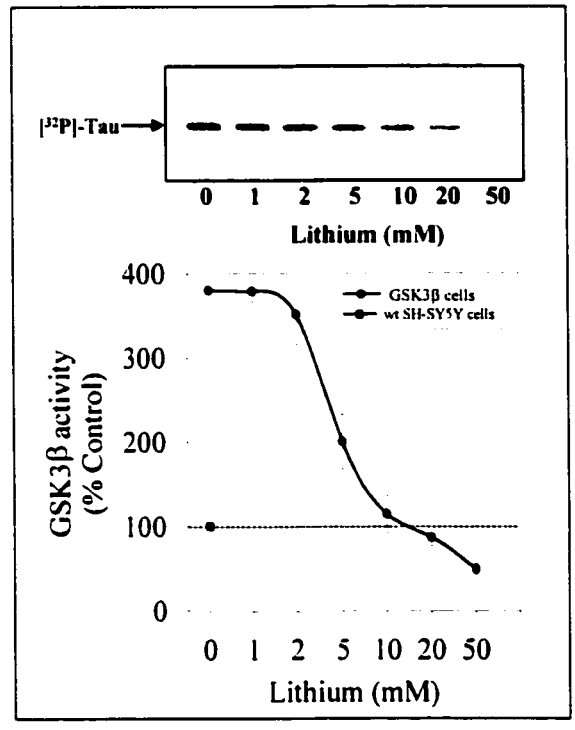



**FIG. 7.** Lithium concentration-dependently inhibits GSK3β activity immunoprecipitated from SH-SY5Y cells overexpressing GSK3β. GSK3β activity was measured by immunoprecipitating GSK3β from serum-starved (24 h) wild-type and GSK3β-overexpressing SH-SY5Y cells; incubating the immunoprecipitated GSK3β with recombinant human tau, [$^{32}$P]ATP, and lithium; and measuring the phosphorylation of tau, as described in Materials and Methods. Quantitative values are expressed as percentages of GSK3β activity in wild-type serum-starved cells. Data are mean ± SEM (bars) values (n = 3).

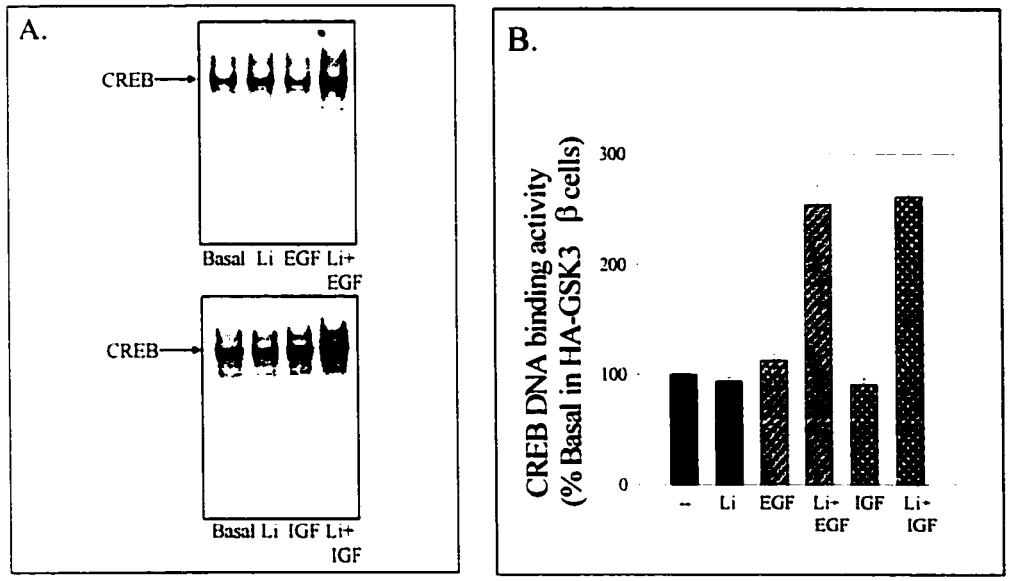



**FIG. 8.** Lithium restores growth factor stimulation of CREB DNA binding activity in SH-SY5Y cells overexpressing GSK3β. **A:** CREB DNA binding activity was measured by EMSA in nuclear extracts prepared from GSK3β-overexpressing cells incubated without serum (24 h) and treated without or with EGF (50 ng/ml for 1 h), or IGF-1 (50 ng/ml for 1 h) alone or following preincubation with lithium (Li; 20 m*M* for 1 h). **B:** Quantitative values are expressed as percentages of basal CREB DNA binding activity in serum-starved GSK3β-overexpressing cells. Data are mean ± SEM (bars) values (n = 4-6).

induced by forskolin in GSK3β-overexpressing cells. To test whether this could result from diminished production of cyclic AMP in these cells, cyclic AMP levels were measured in control and GSK3β-overexpressing cells before and after treatment with 10 μ*M* forskolin. In control SH-SY5Y cells, cyclic AMP levels were increased 4.4-fold by forskolin, from 48 ± 4 to 213 ± 30 nmol/μg protein (n = 7). In contrast, in GSK3β-overexpressing cells the cyclic AMP level was increased only 2.1-fold by forskolin, to 79 ± 1 from a basal level of 38 ± 1 nmol/μg protein (n =7, measurements made in clones 1, 3, and 7 of GSK3β-overexpressing cells). Thus, deficient forskolin-induced CREB activation in GSK3β-overexpressing cells resulted in part from impaired production of cyclic AMP, rather than solely from an inhibitory effect of GSK3β on CREB. Therefore, to examine whether cyclic AMP-induced stimulation of CREB DNA binding activity is inhibited by GSK3β, the cyclic AMP analog, dibutyryl cyclic AMP (diBcAMP), was used in both wild-type and GSK3β-overexpressing SH-SY5Y cells to stimulate CREB DNA binding activity. DiBcAMP stimulated CREB DNA binding activity in wild-type SH-SY5Y cells to 224 ± 16% of the basal activity (Fig. 9). However, diBcAMP stimulation of CREB DNA binding was completely blocked in GSK3β-overexpressing SH-SY5Y cells, but pretreatment of these cells with 20 m*M* lithium restored diBcAMP stimulation of CREB DNA binding activity (Fig. 9). These findings support the conclusion that GSK3β inhibits cAMP-induced activation of CREB DNA binding activity.

To confirm that the facilitation by lithium of CREB DNA binding activity in GSK3β-overexpressing cells was due to its inhibition of GSK3β, another inhibitor of GSK3β, sodium valproate (Chen et al., 1999) was used. In vitro, sodium valproate

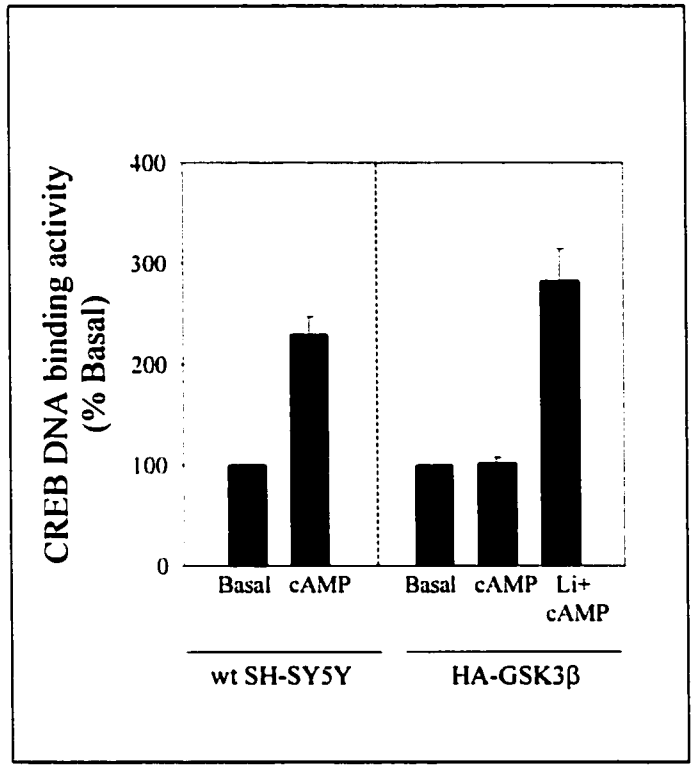



**FIG. 9.** Lithium restores cyclic AMP stimulation of CREB DNA binding activity in SH-SY5Y cells overexpressing GSK3β. CREB DNA binding activity was measured by EMSA in nuclear extracts prepared from GSK3β-overexpressing cells incubated without serum (24 h) and treated without or with diBcAMP (100 μ*M* for 1 h) alone or following preincubation with lithium (20 m*M* for 1 h). Quantitative values are expressed as percentages of basal CREB DNA binding activity in serum-starved GSK3β-overexpressing cells. Data are means ± SEM (bars) values (n = 2-3).

concentration-dependently inhibited GSK3β activity in GSK3β immunoprecipitates from SH-SY5Y cells overexpressing GSK3β (Fig. 10A). In accordance with the results obtained using lithium, pretreatment of GSK3β-overexpressing cells with sodium valproate restored EGF, IGF-1, and diBcAMP stimulation of CREB DNA binding activity (Fig. 10B). These findings support the conclusion that CREB DNA binding activity is negatively regulated by GSK3β and demonstrate that this inhibition is reversible by two inhibitors of GSK3β.

## DISCUSSION

CREB is a key transcription factor involved in several critical functions of the brain, including learning, plasticity, and cell survival (Struthers et al. 1991; Davis et al., 1996; Deisseroth et al., 1996; Silva et al., 1998; Bevilaqua et al., 1999), but the complex signaling systems that regulate the activity of CREB are incompletely defined. Although CREB is known to be activated by several growth factors that activate the PI3K-Akt signaling pathway, which leads to inhibition of GSK3β, it was unclear whether the inhibition of GSK3β by Akt contributes to the regulation of CREB. The results of the present investigation demonstrated with several experimental paradigms that GSK3β is an inhibitory regulator of CREB, that inhibition of GSK3β is necessary for optimal activation of CREB induced by growth factors or cyclic AMP, and that inhibition of GSK3β by lithium can facilitate CREB activation.

Phosphorylation of CREB at serine-133 is required for activation of DNA binding, complex formation with CBP, and transcription, since mutation of this residue results in the complete loss of transcriptional activity and the mutated protein can act as a

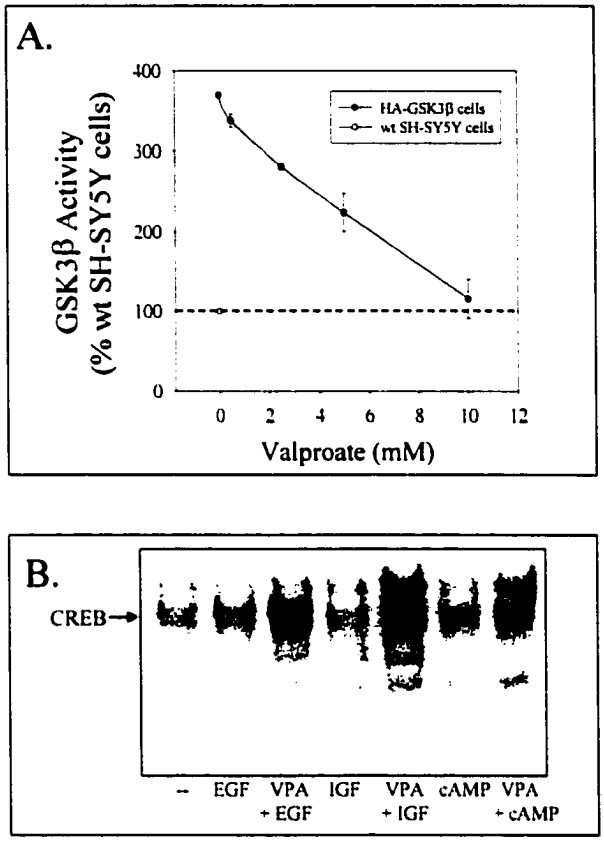



**FIG. 10.** Valproate concentration-dependently inhibits GSK3β activity and restores growth factor and cyclic AMP stimulation of CREB in SH-SY5Y cells overexpressing GSK3β. **A:** GSK3β activity was measured by immunoprecipitating GSK3β from serum-starved (24 h) GSK3β-overexpressing SH-SY5Y cells; incubating the immunoprecipitated GSK3β with recombinant human tau, [$^{32}$P]ATP, and valproate; and measuring the phosphorylation of tau, as described in Materials and Methods. Quantitative values are expressed as percentages of GSK3β activity in serum-starved wild-type SH-SY5Y cells. Data are mean ± SEM (bars) values (n = 3). **B:** CREB DNA binding activity was measured by EMSA in nuclear extracts prepared from GSK3β-overexpressing cells incubated without serum (24 h) and treated without or with EGF (50 ng/ml for 1 h), IGF-1 (50 ng/ml for 1 h), or diBcAMP (100 μ*M* for 1 h) alone or following preincubation with valproate (10 m*M* for 1 h). Quantitative values are expressed as percentages of basal CREB DNA binding activity in serum-starved GSK3β-overexpressing cells. Data are mean ± SEM (bars) values (n = 3).

negative regulator of phospho-CREB (Gonzalez and Montminy, 1989; Habener et al., 1995). Phosphorylation of CREB at serine-133 also creates the consensus sequence, SXXXS(P), for hierarchical phosphorylation by GSK3β of serine-129, whereas in the absence of phosphorylation at serine-133, CREB is not phosphorylated by GSK3β (Fiol et al., 1994; Wang et al., 1994). Many kinases, including protein kinase A, extracellular-related kinases, protein kinase C, Ras-dependent protein kinase, RSK-2, and calcium calmodulin-dependent protein kinase IV, have been shown to activate CREB by phosphorylating serine-133 (Dash et al., 1991; Sheng et al., 1991; Ginty et al., 1994; Xing et al., 1996), thus priming CREB as a substrate for GSK3β-mediated phosphorylation. This raises the question of what effect secondary phosphorylation by GSK3β has on CREB function. One possible function would be for the action of GSK3β to synergize with serine-133 phosphorylation to fully activate CREB. Another possibility would be for phosphorylation by GSK3β to act as a signal for termination of CREB activity. Evidence for each of these opposing actions has been reported in the only two previous investigations of the functional consequences of GSK3β-mediated phosphorylation of CREB. The data of Fiol et al. (1994) indicated that GSK3β-mediated phosphorylation of CREB at serine-129 is necessary for CREB function, because expression of CREB with a mutation of serine-129 to alanine in PC12 cells was refractory to stimulation by cyclic AMP. Furthermore, activation of a Gal4-CREB reporter was greatly induced by cotransfection of GSK3β in F9 cells (Fiol et al., 1994). The findings of Bullock and Habener (1998) also demonstrated the hierarchical phosphorylation of CREB by GSK3β, but they reported an opposite functional outcome of this phosphorylation. Phosphorylation of CREB by protein kinase A increased the

DNA binding of CREB, whereas secondary phosphorylation of primed CREB by GSK3β attenuated protein kinase A stimulation of CREB DNA binding activity, a finding that was attributed to phosphorylation-mediated changes in structure and net charge of CREB (Bullock and Habener, 1998). Bullock and Habener (1998) attributed the conflicting results of Fiol et al. (1994) to methodological differences, although this does not entirely explain the stimulatory effects of GSK3β that were observed. The results reported here agree entirely with those of Bullock and Habener (1998), who concluded that phosphorylation of serine-129 by GSK3β provides inhibitory regulation of CREB DNA binding. This was evident in the present investigation from the increased CREB DNA binding activity following treatments that indirectly inhibit GSK3β through activation of the PI3K/Akt signaling pathway and with direct inhibition of GSK3β, and from the decreased CREB DNA binding activity in cells with increased GSK3β activity that was reversed by inhibition of GSK3β. Thus, these results support the conclusion that phosphorylation of CREB by GSK3β causes inactivation of the transcription factor.

In 1996, lithium, the primary agent used in the treatment of manic-depressive illness, was found to be a direct inhibitor of GSK3β, with an $IC_{50}$ of approximately 2 *mM*, a concentration near its therapeutic range (Klein and Melton, 1996; Stambolic et al., 1996). This finding provided a possible mechanism for the therapeutic action of lithium and initiated the now widespread use of lithium as an effective tool to identify specific actions of GK3β. The further discovery that GSK3β is directly inhibited by sodium valproate (Chen et al., 1999) provided a second agent with which the actions of GSK3β could be verified and added further support to the possibility that inhibition of GSK3β may be therapeutically important in the treatment of bipolar disorder because sodium

valproate is now widely used in the treatment of this illness. Thus, the findings that treatment of cells with lithium or sodium valproate facilitated CREB DNA binding activity support the conclusion that GSK3β is inhibitory. Previous findings of Ozaki and Chuang (1997) are in accordance with this conclusion; they reported that lithium treatment increased CREB DNA binding activity in cerebellar granule cells, although based on the literature available at that time this effect was correlated to effects of lithium on protein kinase C. However, in a similar study Wang et al. (1999) did not observe increased CREB activation in SH-SY5Y cells treated with lithium. This difference is likely attributable to the use by Wang et al. (1999) of cells maintained in serum-containing media, a condition that maintains a low level of GSK3β activity and a condition in which we also found little effect of lithium. Thus, the present study found that lithium increased CREB DNA binding activity only when GSK3β was not adequately inhibited (i.e., in cells maintained in serum-free media and in cells overexpressing GSK3β). Our findings that sodium valproate, which also inhibits GSK3β, also facilitated CREB activation further support the conclusion that GSK3β is inhibitory toward CREB activity. This common action of lithium and sodium valproate further suggests that inhibition of GSK3β and the consequential facilitation of CREB activation may be of therapeutic relevance since it identifies an outcome common to the two most prevalently used agents to treat bipolar disorder.

The inhibitory effect of GSK3β on CREB should be considered in context with other actions of GSK3β and potential outcomes on cell function. GSK3β has been reported to negatively regulate, through phosphorylation, the activity of a diverse array of transcription factors, including AP-1 (Boyle et al., 1991), heat shock factor-1 (He et al.,

1998; Bijur and Jope, 2000), β-catenin (Rubinfeld et al., 1996; Yost et al., 1996), nuclear factor of activated T-cells (Beals et al., 1997), Myc (Henrikkson et al., 1993), and NFκB (Bournat et al., 2000). Thus, the control of GSK3β activity contributes to the regulation of many transcription factors in addition to CREB and, therefore, must be a central figure in the regulation of gene expression. From this, it appears likely that GSK3β-induced down-regulation of these diverse transcription factors likely contributes to the multiple modes of apoptosis that are facilitated by GSK3β (Pap and Cooper, 1998; Bijur et al., 2000; Hetman et al., 2000) and to the protective effect of lithium against a remarkably broad array of insults (reviewed in Jope, 1999). Additional studies are necessary to further understand the role of CREB and other transcription factors regulated by GSK3β in mood disorders and the potential protection by lithium of these proteins that contribute to neuronal plasticity and cell survival.

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## REFERENCES

Beals C. R., Sheridan C. M., Turck C. W., Gardner P., and Crabtree G. R. (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275**, 1930-1933.

Bevilaqua L. R., Cammarota M., Paratcha G., de Stein M. L., Izquierdo I., and Medina J. H. (1999) Experience-dependent increase in cAMP-responsive element binding protein in synaptic and nonsynaptic mitochondria of the rat hippocampus. *Eur. J. Neurosci.* **11**, 3753-3756.

Bijur G. N. and Jope R. S. (2000) Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3β in the regulation of HSF-1 activity. *J. Neurochem.* **75**, 2401-2408.

Bijur G. N., DeSarno P., and Jope R. S. (2000) Glycogen synthase kinase-3β facilitates staurosporine- and heat shock-induced apoptosis. Protection by lithium. *J. Biol. Chem.* **275**,7583-7590.

Bournat J. C., Brown A. M. C., and Soler A. P. (2000) Wnt-1 dependent activation of the survival factor NF-κB in PC12 cells. *J. Neurosci. Res.* **61**, 21-32.

Boyle W. J., Smeal T., Defize L. H., Angel P., Woodgett J. R., Karin M., and Hunter T. (1991) Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* **64**, 573-584.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. *Annal. Biochem.* **72**, 248-254.

Bullock B. P. and Habener J. F. (1998) Phosphorylation of the cAMP response element binding protein CREB by cAMP-dependent protein kinase A and glycogen synthase kinase-3 alters DNA binding affinity, conformation, and increases net charge. *Biochemistry* **37**, 3795-3809.

Cardinaux J. R., Notis J. C., Zhang Q., Vo N., Craig J. C., Fass D. M., Brennan R. G., and Goodman R. H. (2000) Recruitment of CREB binding protein is sufficient for CREB-mediated gene activation. *Mol. Cell. Biol.* **20**, 1546-1552.

Chen G., Huang L. D., Jiang Y. M., and Manji H. K. (1999) The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J. Neurochem.* **72**, 1327-1330.

Chrivia J. C., Kwok R. P., Lamb N., Hagiwara M., Montminy M. R., and Goodman R. H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859.

Chu B., Soncin F., Price B. D., Stevenson M. A., and Calderwood S. K. (1996) Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J. Biol. Chem.* **271**, 30847-30857.

Cross D. A., Alessi D. R., Cohen P., Andjelkovich M., and Hemmings B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.

Dash P. K., Karl K. A., Colicos M. A., Prywes P., and Kandel E. R. (1991) cAMP response element-binding protein is activated by $Ca^{2+}$/calmodulin- as well as cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **88**, 5061-5065.

Datta S. R., Brunet A., and Greenberg M. E. (1999) Cellular survival: a play in three Akts. *Genes Dev.* **13**, 2905-2927.

Davis G. W., Schuster C. M., and Goodman C. S. (1996) Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron* **17**, 669-679.

Deisseroth K., Bito H., and Tsien R. W. (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron* **16**, 89-101.

Embi N., Rylatt D. B., and Cohen P. (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur. J. Biochem.* **107**, 519-527.

Ferkey D. M., and Kimelman D. (2000) GSK-3: new thoughts on an old enzyme. *Dev. Biol.* **225**, 471-479.

Fiol C. J., Haseman J. H., Wang Y. H., Roach P. J., Roeske R. W., Kowalczuk M., and DePaoli-Roach A. A. (1987) Phosphoserine as a recognition determinant for glycogen synthase kinase-3: phosphorylation of a synthetic peptide based on the G-component of protein phosphatase-1. *Arch. Biochem. Biophys.* **267**, 797-802.

Fiol C. J., Williams J. S., Chou C. H., Wang Q. M., Roach P. J., and Andrisanni O. M. (1994) A secondary phosphoryation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in control of gene expression. *J. Biol. Chem.* **269**, 32187-32193.

Ginty D. D., Bonni A., and Greenberg M. E. (1994) Nerve growth factor activates Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell* **77**, 713-725.

Gonzalez G. A. and Montminy M. R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675-680.

Habener J. F., Miller C. P., and Vallejo M. (1995) cAMP-dependent regulation of gene transcription by cAMP response element-binding protein and cAMP response element modulator. *Vitam. Horm.* **51**, 1-57.

He B., Meng Y. H., and Mivechi N. F. (1998) Glycogen synthase kinase 3β and extracellular signal-regulated kinase inactivate heat shock transcription factor 1

by facilitating the disappearance of transcriptionally active granules after heat shock. *Mol. Cell Biol.* **18**, 6624-6633.

Henriksson M., Bakardjiev A., Klein G., and Luscher B. (1993) Phosphorylation sites mapping the N-terminal domain of c-myc modulate its transforming potential. *Oncogene* **8**, 3199-3209.

Hetman M., Cavanaugh J. E., Kimelman D., and Xia Z. (2000) Role of glycogen synthase kinase-3β in neuronal apoptosis induced by trophic withdrawal. *J. Neurosci.* **20**, 2567-2674.

Jean D., Harbison M., McConkey D. J., Ronai Z., and Bar-Eli M. (1998) CREB and its associated proteins act as survival factors for human melanoma cells. *J. Biol. Chem.* **273**, 24884-24890.

Ji L., Mochon E., Arcina M., and Boxer L. M. (1996) CREB proteins function as positive regulators of the translocated bcl-2 allele in t(14;18) lymphomas *J. Biol. Chem.* **271**, 22687-22691.

Jope R. S. (1999) Anti-bipolar therapy: mechanism of action of lithium. *Mol. Psychiat.* **4**, 117-128.

Kim L. and Kimmel A. R. (2000) GSK3, a master switch regulating cell-fate specification and tumorigenesis. *Curr. Opin. Genet. Dev.* **10**, 508-514.

Klein P. S. and Melton D. A. (1996) A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93**, 8455-8459.

Lesort M., Jope R. S., and Johnson G. V. W. (1999b) Insulin biphasically modulates tau phosphorylation: involvement of glycogen synthase kinase-3β and fyn tyrosine kinase. *J. Neurochem.* **72**, 576-584.

Ozaki N. and Chuang D-M. (1997) Lithium increases transcription factor binding to AP-1 and cyclic AMP-responsive element in cultured neurons and rat brain. *J. Neurochem.* **69**, 2336-2344.

Pap M. and Cooper G. M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J. Biol. Chem.* **273**, 19929-19932.

Plyte S. E., Hughes K., Nikolakaki E., Pulverer B. J., and Woodgett J. R. (1992) Glycogen synthase kinase-3: functions in oncogenesis and development. *Biochim. Biophys. Acta* **1114**, 147-162.

Pugazhenthi S., Miller E., Sable C., Young P., Heidenreich K. A., Boxer L. M., and Reusch J. E.-B. (1999) Insulin-like growth factor-I induces bcl-2 promoter

through the transcription factor cAMP-response element binding protein. *J. Biol. Chem.* **274**, 27529-27535.

Riccio A., Ahn S., Davenport C. M., Blendy J. A., and Ginty D. D. (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science* **286**, 2358-2361.

Rubinfeld B., Albert I., Porfiri R., Fiol C., Munemitsu S., and Polakis P. (1996) Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.

Rudolph D., Tafuri A., Gass P., Hammerling G. J., Arnold B., and Schutz G. (1998) Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein. *Proc. Natl. Acad. Sci. USA* **95**, 4481-4486.

Rylatt D. B., Aitken A., Bilham T., Condon G. D., Embi N., and Cohen P. (1980) Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase. *Eur. J. Biochem.* **107**, 529-537.

Sayas C. L., Moreno-Flores M. T., Avila J., and Wandosell F. (1999) The neurite retraction induced by lysophosphatidic acid increases Alzheimer's disease-like Tau phosphorylation. *J. Biol. Chem.* **274**, 37046-37052.

Sheng M., Thomspon M.A., and Greenberg M.E. (1991) CREB: a $Ca^{2+}$-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427-1430.

Silva A. J., Kogan J. H., Franklan P. W., and Kida S. (1998) CREB and memory. *Annu. Rev. Neurosci.* **21**, 127-148.

Stambolic V., Ruel L., and Woodgett J. R. (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* **6**, 1664-1668.

Struthers R. S., Vale W. W., Arias C., Sawchenko P. E., and Montminy M. R. (1991) Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant. *Nature* **350**, 622-624.

Walton M. R. and Dragunow I. (2000) Is CREB a key to neuronal survival? *Trends Neurosci.* **23**, 48-53.

Walton M., Sirimanne E., Williams C., Gluckman P., and Dragunow M. (1996) The role of cyclic AMP-responsive element binding protein (CREB) in hypoxic-ischemic brain damage and repair. *Mol. Brain. Res.* **43**, 21-29.

Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A., and Roach, P. J. (1994) Glycogen synthase kinase-3β is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J. Biol. Chem.* **269**, 14566-14574.

Wang J. F., Asghari V., Rockel C., and Young L. T. (1999) Cyclic AMP responsive element binding protein phosphorylation and DNA binding is decreased by chronic lithium but not valproate treatment of SH-SY5Y neuroblastoma cells. *Neuroscience* **91**, 771-776.

Wilson B. E., Mochon E., and Boxer L. M. (1996) Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Mol. Cell. Biol.* **16**, 5546-5556.

Xing J., Ginty D. D., and Greenberg M. E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**, 959-963.

Yang X. J., Ogryzko V. V., Nishikawa J., Howard B. H., and Nakatani Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**, 319-324.

Yost C., Torres M., Miller J. R., Huang E., Kimelman D., and Moon R. T. (1996) The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443-1454.

# CONCLUSIONS

The studies within this dissertation focused on identifying mechanisms involved in the regulation of transcription factor activity. Transcription factors are considered critical targets of signal transduction events because they are responsible for transferring information received by cells from the extracellular environment to the nucleus to regulate numerous cellular functions. Transcription factors serve multiple critical roles in the cell, acting as filters to limit the magnitude of signals reaching the nucleus, amplifiers to increase the sensitivity of response to certain signals, integrators to prevent the overlapping of conflicting signals, and interpreters to translate diverse types of signals. Fulfilling these functions is the critical responsibility of transcription factors, proteins that, when activated, bind specific DNA sequences to regulate transcription. Thus, the regulation of transcription factors by signaling systems can tremendously affect cell function.

A key cellular signaling system is the phosphoinositide signaling cascade that, in response to external stimuli, transposes signals by generating second messengers that activate protein kinases, initiate intracellular calcium release, and ultimately modulate gene expression (reviewed in Jope and Williams, 1994; Pacheco and Jope, 1996). This modulation of gene expression is often accomplished by regulating the activation state of transcription factors. The first study in this dissertation revealed the robust, rapid, and transient activation of the early growth response-1 (Egr-1) transcription factor in response

to stimulation of cholinergic muscarinic receptors. The Egr-1 transcription factor has recently received great interest because of its sensitive regulation by activation of neuronal signaling and its involvement in the processes of learning and memory (Worley et al., 1991; Fordyce et al., 1994). The present study found that the mood-stabilizing drug valproate reduced signal-induced activation of Egr-1 DNA binding activity. This finding might prove to be a useful tool in future studies to further characterize the role of Egr-1 in neuronal function and to further identify the mechanism of action of valproate on cell functions.

Another perhaps more intriguing signaling intermediate that regulates transcription factor activation is glycogen synthase kinase-3β (GSK3β). Although many individual actions of GSK3β have been reported, these have not been integrated into one comprehensive scheme of the regulatory role of GSK3β on transcription factors. This was accomplished in the second study. Notable aspects of GSK3β are that it is an inhibitory regulator of a diverse array of transcription factors and that most of these transcription factors stimulate the expression of genes critical to cell survival. For instance, GSK3β has been demonstrated to phosphorylate and inhibit AP-1 (Boyle et al., 1991), impair activation of HSF-1 (He et al., 1998; Bijur and Jope, 2000), phosphorylate and inhibit NFκB (Bournat et al., 2000), and phosphorylate β-catenin, preventing its nuclear translocation and regulation of Tcf/Lef-mediated transcription (Yost et al, 1996; Rubinfeld et al., 1996). Each of these transcription factors has been shown to play important roles in promoting cell survival; so, the widespread inhibitory control exerted by GSK3β is likely to affect cell survival.

Throughout the course of the second study, several intriguing questions emerged. For instance, the facts that Wnt signaling is required for neuronal development (McMahon and Bradley, 1990; Patapoutian and Reichardt, 2000) and that Wnt is negatively regulated by GSK3β (Hart et al., 1998; Peifer and Polakis, 2000) raise the question of how the negative regulation of GSK3β, or more specifically, the removal of the negative influence of GSK3β on each of the regulated transcription factors, contributes to cellular development. An additional question for future studies concerns aspects of GSK3β regulation. GSK3β is regulated by three distinct mechanisms: phosphorylation (reviewed in Plyte et al. 1992), protein complex formation (Ikeda et al., 1998; Yamamoto et al., 1998; Yost et al., 1998), and intracellular localization (Diehl et al., 1998; Ragano-Caracciolo et al., 1998). However, it has yet to be addressed how these regulatory mechanisms are integrated. Does phosphorylation of GSK3β affect protein complex formation (e.g., the association of GSK3β with axin or GBP)? Does either phosphorylation or protein complex formation affect the intracellular localization of GSK3β? For example, does phosphorylation of GSK3β affect its nuclear import or export? Another question raised by this study is whether transcription factors exhibit different sensitivities to regulation by GSK3β. From our initial attempt to address this question, results of the second study demonstrated that AP-1, CREB, β-catenin, and Myc display different sensitivities to regulation by GSK3β. Notably, of the four transcription factors examined, CREB was the most sensitive to regulation by GSK3β.

CREB is one of the most widely studied transcription factors in neuronal systems because of its extensive links with neuronal development and learning and memory (Struthers et al., 1991; Davis et al., 1996; Silva et al., 1998). It was surprising that such a

fundamental issue of how CREB is regulated by phosphorylation at serine-129 had not been addressed except in two studies (Fiol et al., 1994; Bullock and Habener, 1998), which established that CREB was subject to hierarchical phosphorylation by GSK3β but left the functional consequence of this phosphorylation unclear. Although CREB is a key component of several neuronal functions, the complex signaling systems that regulate the activity of CREB have not been completely identified. CREB is known to be activated by growth factors that activate the PI3K-Akt signaling pathway (Sheng et al., 1991; Alberts et al., 1994; Ginty et al., 1994; Xing et al., 1996), but it is unknown whether the inhibition of GSK3β by Akt contributes to the regulation of CREB.

The results of the third study demonstrated, through several experimental paradigms, that GSK3β is an inhibitory regulator of CREB, that inhibition of CREB is necessary for optimal activation of CREB induced by growth factors or cyclic AMP, and that inhibition of GSK3β by lithium can facilitate CREB activation. Now that it is clear that GSK3β is an inhibitory regulator of CREB, one possible direction for future studies is to examine particular genes altered by CREB, GSK3β, and lithium to further delineate their influences on cell function.

The finding that lithium and valproate, two structurally diverse drugs that demonstrate efficacy in the treatment of bipolar disorder, inhibit GSK3β (Klein and Melton, 1996; Stambolic et al., 1996; Chen et al., 1999) provides a basis for the concept that GSK3β may be involved in bipolar disorder. The inhibition of GSK3β by lithium, as well as by valproate, occurs at concentrations within the therapeutic range of these drugs, indicating that therapeutic levels of either of these drugs would effectively dampen signaling events mediated by GSK3β. It is reasonable that a mood stabilizer such as

lithium or valproate may act by lessening the magnitude of signals without completely blocking crucial signaling events (reviewed in Jope, 1999). This raises intriguing possibilities: GSK3β may be a critical target for the therapeutic actions of mood stabilizing drugs, GSK3β activity may be altered in psychiatric disorders such as bipolar disorder, and dysfunctions in signaling systems that regulate the activity of GSK3β may contribute to bipolar disorder. Continued study of these possibilities will enhance our knowledge of the role of GSK3β in the underlying biochemical dysfunction of bipolar disorder.

## GENERAL REFERENCES


Alberts A. S., Arias J., Hagiwara M., Montminy M. R., and Feramisco J. R. (1994) Recombinant cyclic AMP response element binding protein (CREB) phosphorylated on Ser-133 is transcriptionally active upon its introduction into fibroblast nuclei. *J. Biol. Chem.* **269**, 7623-7630.

Asghari V., Wang J. F., Reiach J. S., and Young L. T. (1998) Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. *Mol. Brain Res.* **58**, 95-102.

Beals C. R., Sheridan C. M., Turck C. W., Gardner P., and Crabtree G. R. (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275**, 1930-1933.

Beckmann A.M. and Wilce P.A. (1997) Egr transcription factors in the nervous system. *Neurochem. Int.* **4**, 477-510.

Berridge M. J., Downes C. P., and Hanley M. R. (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.* 206, 587-595.

Bijur G. N. and Jope R. S. (2000) Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3β in the regulation of HSF-1 activity. *J. Neurochem.* **75**, 2401-2408.

Bournat J. C., Brown, A. M. C., and Soler, A. P. (2000) Wnt-1 dependent activation of the survival factor NF-κB in PC12 cells. *J. Neurosci. Res.* **61**, 21-32.

Boyle W. J., Smeal T., Defize L. H., Angel P., Woodgett J. R., Karin M., and Hunter T. (1991) Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* **64**, 573-584.

Brindle P. K. and Montminy M. R. (1992) The CREB family of transcription activators. *Curr. Opin. Genet. Dev.* **2**, 199-204.

Bullock B. P. and Habener J. F. (1998) Phosphorylation of the cAMP response element binding protein CREB by cAMP-dependent protein kinase A and glycogen synthase kinase-3 alters DNA-binding affinity, conformation, and increases net charge. *Biochemistry* **37**, 3795-3809.

Chen G., Huang L. D., Jiang Y. M., and Manji H. K. (1999) The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J. Neurochem.* **72**, 1327-1330.

Chrivia J. C., Kwok R. P., Lamb N., Hagiwara M., Montminy M. R., and Goodmand R. H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859.

Davis G. W., Schuster C. M., and Goodman C. S. (1996) Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron* **17**, 669-679.

de Groot R. P., Auwerx J., Bourouis M., and Sassone-Corsi P. (1993) Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the *Drosophila* kinase shaggy. *Oncogene* **7**, 841-847.

Deisseroth K., Bito H., and Tsien R. W. (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron* **16**, 89-101.

Diehl J. A., Cheng M., Roussel M. F., and Sherr C. J. (1998) Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12**, 3499-3511.

Dixon J.F. and Hokin L.E. (1997) The antibipolar drug valproate mimics lithium in stimulating glutamate release and inositol 1,4,5-trisphosphorate accumulation in brain cortex slices but not accumulation of inositol monophosphates and bisphosphates. *Proc. Natl. Acad. Sci. USA* **94**, 4757-4760.

Ebihara T. and Saffen D. (1997) Muscarinic acetylcholine receptor-mediated induction of zif268 mRNA in PC12D cells requires protein kinase C and the influx of extracellular calcium. *J. Neurochem.* **68**, 1001-1010.

Embi N., Rylatt D. B., and Cohen P. (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur. J. Biochem.* **107**, 519-527.

Fiol C. J., Williams J. S., Chou C. H., Wang Q. M., Roach P. J., and Andrisani O. M. (1994) A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. *J. Biol. Chem.* **269**, 32187-32193.

Fordyce D.E., Bhat R.V., Baraban J.M., and Wehner J.M. (1994) Genetic and activity-dependent regulation of zif268 expression: association with spatial learning. *Hippocampus* **4**, 559-568.

Ginty D. D., Bonni A., and Greenberg M. E. (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell* **77**, 713-725.

Gonzalez G. A., Yamamoto K. K., Fischer W. H., Karr D., Menzel P., Biggs W., Vale W. W., and Montminy M. R. (1989) A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* **337**, 749-752.

Graef I. A., Mermelstein P. G., Stankunas K., Neilson J. R., Deisseroth K., Tsien R. W., and Crabtree G. R. (1999) L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* **401**, 703-708.

Hallcher L. M. and Sherman W. R. (1980) The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. *J. Biol. Chem.* **255**, 10896-10901.

Hart M. J., de los Santos R., Albert I. N., Rubinfeld B., and Polakis P. (1998) Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β. *Curr. Biol.* **8**, 573-581.

He B., Meng Y. H., and Mivechi N. F. (1998) Glycogen synthase kinase 3β and extracellular signal-regulated kinase inactivate heat shock transcription factor 1 by facilitating the disappearance of transcriptionally active granules after heat shock. *Mol. Cell Biol.* **18**, 6624-6633.

Hedgepeth C. M., Conrad L. J., Zhang J., Huang H. C., Lee V. M., and Klein P. S. (1997) Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. *Dev. Biol.* **185**, 82-91.

Hemmings B. A., Aitken A., Cohen P., Rymond M., and Hofmann F. (1982) Phosphorylation of the type-II regulatory subunit of cyclic-AMP-dependent protein kinase by glycogen synthase kinase 3 and glycogen synthase kinase 5. *Eur. J. Biochem.* **127**, 473-481.

Henrikkson M., Bakardjiev A., Klein G., and Luscher B. (1993) Phosphorylation sites mapping the N-terminal domain of c-myc modulate its transforming potential. *Oncogene* **8**, 3199-3209.

Hoshi M., Takashima A., Noguchi K., Murayama M., Sato M., Kondo S., Saitoh Y., Ishiguro K., Hoshino T., and Imahori K. (1996) Regulation of mitochondrial pyruvate dehydrogenase activity by tau protein kinase I/glycogen synthase kinase 3β in brain. *Proc. Natl. Acad. Sci. USA* **93**, 2719-2723.

Ikeda S., Kishida S., Yamamoto H., Murai H., Koyama S., and Kikuchi A. (1998) Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. *EMBO J.* **17**, 1371-1384.

Johnson G. V. W. and Hartigan J. A. (1998) Tau protein in normal and Alzheimer's disease brain: an update. *Alzheimer's Dis. Rev.* **3**, 125-141.

Jope R. S. (1999) Anti-bipolar therapy: mechanism of action of lithium. *Mol. Psychiat.* **4**, 117-128.

Jope R. S. and Song L. (1997) AP-1 and NFκB stimulated by carbachol in human neuroblastoma SH-SY5Y cells are differentially sensitive to inhibition by lithium. *Mol. Brain Res.* **50**, 171-180.

Jope R. S. and Williams M. B. (1994) Lithium and brain signal transduction systems. *Biochem. Pharmacology* **47**, 429-441.

Klein P. S. and Melton D. A. (1996) A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93**, 8455-8459.

Leroy K. and Brion J-P. (1999) Developmental expression and localization of glycogen synthase kinase-3β in rat brain. *J. Chem. Neuroanat.* **16**, 279-293.

McMahon A. P. and Bradley A. (1990) The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

Montminy M. R. and Bilezikjian L. M. (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328**, 175-178.

Nikolakaki E., Coffer P. J., Hemelsoet R., Woodgett J. R., and Defize L. H. (1993) Glycogen synthase kinase 3 phosphorylates Jun family members in vitro and negatively regulates their transactivating potential in intact cells. *Oncogene* **8**, 833-840.

Ozaki N. and Chuang D.M. (1997) Lithium increases transcription factor binding to AP-1 and cyclic AMP response element in cultured neurons and rat brain. *J. Neurochem.* **69**, 2336-2344.

Pacheco M. A. and Jope R. S. (1996) Phosphoinositide signaling in human brain. *Prog. Neurobiol.* **50**, 255-73.

Patapoutian A. and Reichardt L. F. (2000) Roles of Wnt proteins in neural development and maintenance. *Curr. Opin. Neurobiol.* **10**, 392-399.

Peifer M. and Polakis P. (2000) Wnt signaling in oncogenesis and embryogenesis: a look outside the nucleus. *Science* **287**, 1606-1609.

Plyte S. E., Hughes K., Nikolakaki E., Pulverer B. J., and Woodgett J. R. (1992) Glycogen synthase kinase-3: functions in oncogenesis and development. *Biochim. Biophys. Acta* **1114**, 147-162.

Ragano-Caracciolo M., Berlin W. K., Miller M. W., and Hanover J. A. (1998) Nuclear glycogen and glycogen synthase kinase 3. *Biochem. Biophys. Res. Commun.* **249**, 422-427.

Rubinfeld B., Albert I., Porfiri R., Fiol C., Munemitsu S., and Polakis P. (1996) Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.

Rupprecht H. D., Sukhatme V. P. Rupprecht A. P., Sterzel R. B., and Coleman D. L. (1994) Serum response elements mediate protein kinase C dependent transcriptional induction of early growth response hene-1 by arginine vasopressin in rat mesangial cells. *J. Cell Physiol.* **159**, 311-323.

Sachs G. S. and Thase M. E. (2000) Bipolar disorder therapeutics: maintenance treatment. *Biol. Psychiat.* **48**, 573-581.

Sheng M., Thompson M. A., and Greenberg M. E. (1991) CREB: a $Ca^{2+}$-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427-1430.

Silva A. J., Kogan J. H., Frankland P. W., and Kida S. (1998) CREB and memory. *Annu. Rev. Neurosci.* **21**, 127-148.

Stambolic V., Ruel L., and Woodgett J. R. (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* **6**, 1664-1668.

Struthers R. S, Vale W. W., Arias C., Sawchenko P. E., and Montminy M. R. (1991) Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant. *Nature* **350**, 622-624.

Stubbs E. B. and Agranoff B. W. (1993) Lithium enhances muscarinic receptor-stimulated CDP-diacylglycerol formation in inositol-depleted SK-N-SH neuroblastoma cells. *J. Neurochem.* **60**, 1292-1299.

Taniuchi M., Johnson E. M. Jr., Roach P. J., and Lawrence J. C. Jr. (1986) Phosphorylation of nerve growth factor receptor proteins in sympathetic neurons and PC12 cells. In vitro phosphorylation by the cAMP-independent protein kinase FA/GSK-3. *J. Biol. Chem.* **261**, 13342-11349.

Waeber G. and Habener J. F. (1991) Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3',5'-monophosphate response element-binding protein CREB. *Mol. Endocrinol.* **5**, 1431-1438.

Wang Q. M., Fiol C. J., DePaoli-Roach A. A., and Roachj P. J. (1994) Glycogen synthase kinase-3β is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J. Biol. Chem.* **269**, 14566-14574.

Worley P. F., Christy P. A., Nakabeppu Y., Bhat R. V., Cole A. J., and Baraban J. M. (1991) Constitutive expression of zif268 in neocortex is regulated by synaptic activity. *Proc. Natl. Acad. Sci. USA* **88**, 5106-5110.

Xing J., Ginty D. D., and Greenberg M.E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**, 959-963.

Yamamoto H., Kishida S., Uochi T., Ikeda S., Koyama S., Asashima M., and Kikuchi A. (1998) Axil, a member of the Axin family, interacts with both glycogen synthase kinase 3β and β-catenin and inhibits axis formation of Xenopus embryos. *Mol. Cell. Biol.* **18**, 2867-2875.

Yost C., Farr G. H., Pierce S. B., Ferkey D. M., Chen M. M., and Kimelman D. (1998) GBP, an inhibitor of GSK-3, is implicated in Xenopus development and oncogenesis. *Cell* **93**, 1031-1041.

Yost C., Torres M., Miller J. R., Huang E., Kimelman D., and Moon R. T. (1996) The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443-1454.

Yuan P-X., Chen G., Huang L-D., and Manji H. K. (1998) Lithium stimulates gene expression through the AP-1 transcription factor pathway. *Molec. Brain Res.* **58**, 225-230.

Yun Y. D., Dumoulin M., and Habener J.F. (1990) DNA-binding and dimerization domains of adenosine 3',5'-cyclic monophosphate-responsive protein CREB reside in the carboxyl-terminal 66 amino acids. *Mol. Endocrinol.* **4**, 931-939.

**GRADUATE SCHOOL**
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