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Inositol synthesis regulates activation of GSK-3 α in neuronal cells

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Abstract

The synthesis of inositol provides precursors of inositol lipids and inositol phosphates that are pivotal for cell signaling. Mood-stabilizers lithium and valproic acid (VPA), used for treating bipolar disorder, cause cellular inositol depletion, which has been proposed as a therapeutic mechanism of action of both drugs. Despite the importance of inositol, the requirement for inositol synthesis in neuronal cells is not well understood. Here, we examined inositol effects on proliferation of SK-N-SH neuroblastoma cells. The essential role of inositol synthesis in proliferation is underscored by the findings that exogenous inositol was dispensable for proliferation, and inhibition of inositol synthesis decreased proliferation. Interestingly, the inhibition of inositol synthesis by knocking down *INO1*, which encodes inositol-3-phosphate synthase, the rate-limiting enzyme of inositol synthesis, led to inactivation of GSK-3 α by increasing the inhibitory phosphorylation of this kinase. Similarly, the mood-stabilizer VPA effected transient decreases in intracellular inositol, leading to inactivation of GSK-3 α . As GSK-3 inhibition has been proposed as a likely therapeutic mechanism of action, the finding that inhibition of inositol synthesis results in inactivation of GSK-3 α suggests a unifying hypothesis for mechanism of mood-stabilizing drugs.

Keywords

Inositol; glycogen synthesis kinase; valproic acid; bipolar disorder; inositol depletion; myo-inositol-3-phosphate synthase

Introduction

Inositol, a six-carbon cyclitol, is an essential metabolite. It serves as the precursor of inositol lipids and inositol phosphates (Carman and Han, 2011; Henry et al., 2012; Michell, 2008; Michell, 2011), which play crucial roles in gene expression, signal transduction, lipid signaling, vesicle trafficking, and membrane biogenesis (De Camilli et al., 1996; Lemmon, 2003; Majerus and York, 2009; Michell, 2013; Shen et al., 2003; Steger et al., 2003; van Meer et al., 2008). Many types of cultured mammalian cells require supplementation of exogenous inositol for growth, and inositol-requiring mammalian cells and mutants of yeast

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conflict of interest disclosure

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undergo cell death in response to inositol deprivation (Culbertson and Henry, 1975; Eagle, 1955; Eagle et al., 1957; Kao and Puck, 1968; Keith et al., 1977).

Eukaryotic organisms can potentially obtain inositol from the environment, by the *de novo* synthesis of inositol from glucose, or via recycling of inositol by dephosphorylation of inositol phosphates. These processes are orchestrated to maintain intracellular inositol homeostasis. Inositol uptake in yeast (Lai et al., 1995; Lai and McGraw, 1994) and mammals (Wolfson et al., 2000; Wolfson et al., 1998), and inositol biosynthesis in yeast (Henry et al., 2014; Hirsch and Henry, 1986; Loewen et al., 2004) are affected by exogenous inositol. In mammals, inositol uptake is also regulated in response to glucose, pH, osmolality, growth factors, and other stimuli (Di Daniel et al., 2009; Fu et al., 2012; Miyakawa et al., 1999; Novak et al., 1999; Olgemoller et al., 1993; Spizz and Pike, 1992; Uldry et al., 2004; Yorek et al., 1998). Inositol *de novo* synthesis is a highly conserved pathway that is carried out in two steps, of which the conversion of glucose-6-phosphate to inositol-3-phosphate, catalyzed by the *INO1* gene product inositol-3-phosphate synthase (EC 5.5.1.4), is rate-limiting (Eisenberg, 1967; Kindl and Hoffmann-Ostenhof, 1964; Loewus and Kelly, 1962a; Loewus and Kelly, 1962b; Strausberg et al., 2002). The regulation of inositol biosynthesis has been intensively studied in yeasts (Bachhawat et al., 1995; Carman and Han, 2011; Chen et al., 2007; Henry et al., 2012; Loewen et al., 2004; Ye et al., 2013). In addition to the transcriptional regulation of *INO1* in response to exogenous inositol (Henry et al., 2014; Hirsch and Henry, 1986; Loewen et al., 2004), optimal inositol biosynthesis requires glycogen synthase kinase-3 (GSK-3) (Azab et al., 2007) and inositol pyrophosphates (Ye et al., 2013). Furthermore, Ino1 is posttranslationally regulated by phosphorylation (Deranieh et al., 2013), and enzyme activity is inhibited by the glycolysis intermediate dihydroxyacetone phosphate (DHAP) (Migaud and Frost, 1996; Shi et al., 2005). Mammalian *INO1* expression is altered by estrogen, glucose, and lovastatin, and is regulated by the transcription factor E2F1 (Guan et al., 2003; Rivera-Gonzalez et al., 1998; Seelan et al., 2004; Seelan et al., 2011). Highly regulated inositol synthesis underscores the importance of maintaining inositol homeostasis.

The brain maintains a high level of free inositol (5–50 mM), which is about 100 times higher than that in blood and other tissues (Palmano et al., 1977; Sherman et al., 1977; Stokes et al., 1983; Wong et al., 1987). Altered inositol levels in the brain are associated with psychiatric and neurological problems (Seelan et al., 2009; Shi et al., 2006). For example, levels of inositol are altered in the brains of patients with Down syndrome (Acevedo et al., 1997; Berry et al., 1995), stroke (Rumpel et al., 2003), bipolar disorder (Belmaker et al., 2002; Shimon et al., 1997), and suicide victims (Shimon et al., 1997). Although dietary inositol can cross the blood-brain barrier and enter the cerebrospinal fluid and brain parenchyma, this process is very slow (Aukema, 1994; Spector and Lorenzo, 1975). Inositol levels in the brain primarily depend on inositol recycling and *de novo* synthesis (Williams et al., 2002). Interestingly, brain phosphatidylinositol levels are not affected when inositol uptake is blocked in inositol transporter-deficient mice (Berry et al., 2004), suggesting that inositol synthesis may be important for the synthesis of inositol lipids. However, the requirement of brain cells for inositol synthesis and the cellular consequences of perturbation of inositol synthesis in neuronal cells are not well studied.

Lithium, a mood-stabilizer used for the treatment of bipolar disorder, is an uncompetitive inhibitor of inositol monophosphatase and inositol polyphosphatase (Allison and Stewart, 1971; Berridge et al., 1989; Hallcher and Sherman, 1980; Pollack et al., 1994), and causes a decrease in intracellular inositol by blocking inositol recycling and synthesis. The mood-stabilizer valproic acid (VPA) inhibits inositol biosynthesis by indirectly decreasing activity of the rate-limiting enzyme Ino1 (Ju and Greenberg, 2003; Shaltiel et al., 2004; Vaden et al., 2001). Both drugs decrease cellular inositol and inositol 1,4,5-trisphosphate levels (Eickholt et al., 2005; Shimshoni et al., 2007; Williams et al., 2002), indicating that inositol depletion may attenuate inositol-dependent signaling. While inositol depletion may be therapeutically relevant (Berridge et al., 1989), another proposed target of mood-stabilizers is GSK-3 (Klein and Melton, 1996; Lucas and Salinas, 1997). The two major isoforms of GSK-3 (EC 2.7.11.1 and EC 2.7.11.26), GSK-3 α and GSK-3 β , share 85% sequence homology and are encoded by independent genes (Cohen and Frame, 2001). Both isoforms are expressed in the brain and have many regulatory functions in neural systems, including neurogenesis, neuronal structure, synaptic plasticity, and neuronal survival (Hur and Zhou, 2010). Lithium is a competitive inhibitor of GSK-3, and lithium treatment increases the inhibitory phosphorylation of this kinase (Klein and Melton, 1996; Lucas and Salinas, 1997; Ryves and Harwood, 2001; Zhang et al., 2003). Lithium-induced GSK-3 phosphorylation is caused by disrupting the signaling complex of Akt, β -arrestin 2, and protein phosphatase 2A (Beaulieu et al., 2008). Previous studies have not linked lithium-mediated inhibition of GSK-3 to inositol depletion. Interestingly, however, the inositol-depleting drug VPA has also been shown to inhibit GSK-3 activity (Chen et al., 1999; Chen et al., 2006; De Sarno et al., 2002; Kim et al., 2005). Common cellular effects of inositol depletion and GSK-3 inhibition in response to lithium and VPA treatment suggest that inositol metabolism and GSK-3 activity may be interdependent. It has not been determined if inositol metabolism affects GSK-3 activity in neuronal cells. Elucidating the interplay between inositol synthesis and GSK-3 in neuronal cells will have important implications for the pathophysiologic basis of a wide range of disorders in which inositol levels play a role.

In the current study, we characterized the role of inositol synthesis in proliferation of SK-N-SH neuroblastoma cells. We found that *INO1* expression is essential for cell proliferation and neurite outgrowth. We further showed that inositol synthesis regulates GSK-3 α activation, as inhibition of inositol synthesis by knocking down *INO1* expression or exposure to VPA leads to increased phosphorylation at Ser21 of GSK-3 α , which inactivates the kinase. Interestingly, inositol depletion caused by starving for exogenous inositol did not affect proliferation and GSK-3 phosphorylation. This is the first demonstration in neuronal cells of the importance of inositol *de novo* synthesis, and the first report showing that inositol synthesis affects GSK-3 α activation. These findings have implications for the therapeutic mechanisms of mood-stabilizers and suggest that inositol synthesis and GSK-3 activity are intrinsically related.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), Medium 199, and penicillin-streptomycin solution (100X) were purchased from Invitrogen. Fetal bovine serum (FBS) and dialyzed FBS were purchased from Hyclone. Inositol, lithium chloride, valproic acid, glucose-6-phosphate, NAD⁺, inositol dehydrogenase, and diaphorase were purchased from Sigma. Control shRNA lentiviral particles and *INO1* (also named ISYNA1) shRNA lentiviral particles, puromycin dihydrochloride, and polybrene were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The protease inhibitors and phosphatase inhibitors were purchased from Roche.

The rabbit polyclonal IgG against the Ino1 protein (H-300), mouse monoclonal IgG against actin (C-2), and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit monoclonal IgG against phospho-GSK-3 α (ser21) (36E9), rabbit monoclonal IgG against GSK-3 α (D80E6), rabbit monoclonal IgG against phospho-GSK-3 β (Ser9) (D85E12), rabbit monoclonal IgG against GSK-3 β (27C10), and anti-rabbit IgG were purchased from Cell Signaling Technology. All the primary antibodies were diluted 1:1000 and secondary antibodies 1:5000 in dilution buffer containing 1X tris-buffered saline (TBS), 0.1% Tween-20 with 5% nonfat dry milk.

Cell culture

SK-N-SH neuroblastoma cells were obtained from ATCC. Cells were regularly cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For experiments to test effects of inositol deficiency, Medium 199 supplemented with 10% dialyzed FBS was used as inositol-deficient media. Inositol was added to this medium where indicated for inositol-rich media. All cells were cultured at 37°C in 95% air and 5% CO₂. Of note, DMEM contains 40 μ M inositol, and Medium 199 has 0.28 μ M inositol. The dialyzed FBS contains trace amounts of small molecules.

Establishment of stable cell lines

SK-N-SH cells were transduced with lentiviral particles that contain 3 specific constructs targeting human *INO1*, or with control lentiviral particles containing non-specific scrambled shRNA. Cells were grown for 24 hours after the transduction, and stable cells were selected after culture in fresh media with 5 μ g/ml puromycin for 2 weeks. The knockdown efficiency was determined by measuring Ino1 protein levels in the knockdown cells compared to levels in the control cells.

Proliferation assay

Cell proliferation was carried out using a proliferation assay kit (CellTiter 96 AQueous One Solution cell proliferation assay, Promega) and following the manufacturer's instruction. Cells were inoculated at a concentration of 5,000 cells per well in 96-well plates. On the indicated days, the assay reagent containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)] was added and incubated at 37°C for 4 hours. Relative cell numbers were quantified

by absorbance at 490 nm. The quantity of MTS formazan produced from MTS tetrazolium is directly proportional to the number of living cells (Cory et al., 1991).

RNA isolation and real-time quantitative PCR (RT-qPCR)

SK-N-SH cells were cultured in 6-well plates. Total RNA was extracted using the RNeasy Mini Plus kit (QIAGEN, Valencia, CA). Complementary DNA (cDNA) was synthesized using the First Strand cDNA synthesis Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's manuals. RT-qPCR reactions were performed in a 20 μ L volume using Brilliant III Ultra-Faster SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA). Triplicates were included for each reaction. The primers for RT-qPCR are listed in Table 1. RNA levels were normalized to succinate dehydrogenase, SDHA. Relative values of mRNA transcripts are shown as fold change relative to indicated controls.

SDS-PAGE and Western blots

Cell extracts were obtained by breaking cells in lysis buffer containing 50 mM Tris, 125 mM sodium chloride, 1% NP-40, 2 mM EDTA, 1x protease inhibitor cocktail, and 1x phosphatase inhibitor cocktail and were clarified twice by 10 min-centrifugation at 13,000 g at 4°C to remove cell debris. Protein concentration was determined using the BCA™ protein assay (Pierce Protein), with bovine serum albumin as the standard. Cell extracts containing 20 μ g protein were boiled with protein gel sample buffer, separated on 10% SDS-PAGE, and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated with antibodies and visualized using ECL substrate (Pierce Protein). ImageJ software was used to quantify the intensities of bands.

Measurement of intracellular inositol

Intracellular inositol was measured as described previously (Ju and Greenberg, 2003; Ye et al., 2013) with minor modifications. Briefly, cell extracts were obtained in lysis buffer containing 50 mM Tris, 125 mM sodium chloride, 1% NP-40, 2 mM EDTA and were clarified twice by 10 min-centrifugation at 13,000 g at 4°C to remove cell debris. Cell extracts containing 50 μ g protein were used to measure intracellular inositol. Protein was precipitated using ice-cold 7.5% perchloric acid. After centrifugation, perchloric acid in the supernatants was removed by titration to pH 7.0 with ice-cold 10 M potassium hydroxide. The cell extracts were again clarified by centrifugation for 5 min at 2,000 g at 4°C. The supernatants were collected, and intracellular inositol was measured by enzyme-coupled fluorescence assay (Maslanski and Busa, 1990). Inositol content in cell extracts of 50 μ g protein was normalized to the indicated controls.

Statistical Analysis

The data were presented as the mean \pm SD. The *p* value was calculated by two-tailed Student's *t*-test, and differences were considered significant when *p* < 0.05. All experiments were carried out in at least triplicates.

RESULTS

Exogenous inositol is not essential for cell proliferation or maintaining inositol homeostasis in SK-N-SH cells

Inositol is an essential growth factor that is required for survival and proliferation of many types of cultured cells (Eagle et al., 1957). Inositol deficiency in these cells causes an arrest of cell growth, cytopathogenic defects, and cell death. However, some cells are able to proliferate in inositol-free or inositol-deficient culture media due to active inositol biosynthesis (Eagle et al., 1957). To determine if inositol is essential for SK-N-SH neuronal cells, we assayed cell growth in inositol-deficient media (Medium 199 with 10% dialyzed serum). Medium 199 contains only 0.28 μM inositol, which is significantly less than the minimal requirement (1 μM) reported for most types of cells (Eagle et al., 1957). As seen in Fig. 1A, proliferation of cells cultured in the inositol-deficient media was similar to that of cells grown in media supplemented with exogenous inositol. The ability of SK-N-SH cells to grow in inositol-deficient media indicates that the *de novo* synthesis of inositol provides sufficient inositol for cell proliferation. Interestingly, cells cultured in inositol-deficient media exhibited levels of intracellular inositol similar to those of cells grown in media supplemented with 0.5–10 mM inositol (Fig. 1B). The homeostatic inositol pool in SK-N-SH cells suggests that inositol biosynthesis may be upregulated in inositol-deficient media.

Inositol biosynthesis is essential for cell proliferation

To understand if inositol biosynthesis is required for SK-N-SH cell growth, we decreased inositol biosynthesis by stably knocking down expression of the gene encoding inositol-3-phosphate synthase, *INO1*, which encodes the rate-limiting enzyme of inositol biosynthesis. As shown in Fig. 2A, *INO1* knockdown cells exhibited a 90% decrease in Ino1 protein levels. As expected, *INO1* knockdown resulted in a profound decrease in intracellular inositol levels (Fig. 2B). Cell proliferation was significantly decreased in *INO1* knockdown cells (Fig. 2C). In addition, neurite outgrowth was remarkably inhibited in cells in which *INO1* expression was decreased (Fig. 2D). These findings indicate that *de novo* inositol synthesis is essential for cell proliferation and neurite outgrowth in SK-N-SH cells.

Because *INO1* knockdown causes inositol depletion leading to decreased proliferation, we questioned if the defect might be rescued by exogenous inositol or exacerbated by the inositol-depleting drugs, lithium and VPA. Unexpectedly, neither exogenous inositol nor lithium and VPA affected proliferation in control and *INO1* knockdown cells (Fig. 2C). To determine if exogenous inositol restores the decreased intracellular inositol in *INO1* knockdown cells, control and *INO1* knockdown cells cultured in DMEM supplemented with 10% serum were replenished with inositol-rich (containing 1 mM inositol) or inositol-deficient media for 5 hours. As shown in Fig. 3A, in inositol-deficient media, inositol levels in *INO1* knockdown cells were only 20% of those in control cells, whereas inositol in *INO1* knockdown cells cultured in inositol-rich media was similar to the control levels. While exogenous inositol was able to restore the inositol depletion caused by *INO1* knockdown, it was not able to rescue the proliferation defect (Fig. 2C). It is likely that the regulation of inositol synthesis may provide a pool of inositol functionally distinct from imported inositol, which is important for proliferation.

Exogenous inositol does not regulate transcription of genes for inositol synthesis and uptake, *INO1*, *SMIT1*, and *HMIT*

In yeast cells, exogenous inositol modulates the biosynthesis and uptake of inositol by controlling transcription of the inositol biosynthetic gene *INO1* (Henry et al., 2014; Hirsch and Henry, 1986; Loewen et al., 2004) and the genes encoding inositol transporters (Lai et al., 1995; Lai and McGraw, 1994). To ascertain if this is a conserved mechanism regulating inositol metabolism in SK-N-SH cells, we determined the effects of exogenous inositol on mRNA levels of *INO1*, Na⁺/inositol transporter *SMIT1*, and H⁺/inositol transporter *HMIT* in these cells. Cells were initially cultured in inositol-deficient media to deplete inositol. After supplementation with inositol (0.1, 1, 10 mM) for the indicated times, cells were harvested for mRNA analysis. As shown in Fig. 3B, mRNA levels of human *INO1* were not affected by the addition of inositol. Consistent with this finding, *INO1* protein levels were also not altered by exogenous inositol (Fig. 3B). In addition, the genes encoding inositol transporters were not regulated by inositol, as mRNA levels of *SMIT1* (Fig. 3C) and *HMIT* (Fig. 3D) were not significantly changed in response to exogenous inositol. Therefore, in contrast to regulation of inositol biosynthesis in yeast cells, the biosynthesis and uptake of inositol were not transcriptionally regulated in response to exogenous inositol in SK-N-SH cells.

Decreased *Ino1* leads to inactivation of *GSK3 α*

Previous studies showed that lithium and VPA inhibit inositol synthesis (Allison and Stewart, 1971; Berridge et al., 1989; Hallcher and Sherman, 1980; Ju and Greenberg, 2003; Pollack et al., 1994; Shaltiel et al., 2004; Vaden et al., 2001) and GSK-3 activity (Chen et al., 1999; Chen et al., 2006; De Sarno et al., 2002; Kim et al., 2005; Klein and Melton, 1996; Lucas and Salinas, 1997). To address the possibility that inositol synthesis affects GSK-3 activity, we measured levels of phosphorylation at Ser21 of GSK-3 α and at Ser9 of GSK-3 β in SK-N-SH cells as a function of inositol depletion. Phosphorylation of these sites inactivates the kinase activity of GSK-3 (Cross et al., 1995; Srivastava and Pandey, 1998). As seen in Fig. 4A, GSK-3 α phosphorylation was increased in response to inhibition of inositol synthesis, while GSK-3 β was not significantly altered. GSK-3 α phosphorylation was dependent on the level of *Ino1* knockdown. Thus, an 82% decrease in *Ino1* protein in shRNA-*INO1*-2 cells led to a 2.4-fold increase in GSK-3 α phosphorylation, while a 60% decrease in shRNA-*INO1*-1 led to a 1.5-fold increase. These results indicated that decreasing the *de novo* synthesis of inositol led to GSK-3 α inactivation.

To investigate if GSK-3 α inactivation resulting from *INO1* knockdown is due to inositol depletion, we determined the effects of exogenous inositol on GSK-3 phosphorylation. While intracellular inositol was significantly decreased by starving for exogenous inositol (Fig. 3A), GSK-3 phosphorylation remained similar in cells cultured in the absence or presence of inositol (Fig. 3C). Therefore, it is the inhibition of *Ino1*-catalyzed inositol synthesis, but not the resulting inositol depletion, that leads to the inactivation of GSK-3 α .

VPA-induced transient decrease in inositol is associated with GSK-3 α inactivation

We have previously shown that the mood stabilizer VPA inhibits inositol synthesis in both yeast and human cells (Ju and Greenberg, 2003; Shaltiel et al., 2004; Vaden et al., 2001). If

inhibition of inositol synthesis was the cause of inhibitory phosphorylation of GSK-3 α as seen in Fig. 4A, VPA may be expected to cause a similar effect. To address this possibility, we assayed GSK-3 phosphorylation in cells treated with VPA. As seen in Fig. 4B, VPA caused a significant decrease in inositol levels in the first hour of exposure, after which inositol levels were restored, probably as a result of recycling from inositol phosphates. As predicted, GSK-3 α phosphorylation was increased in response to VPA (Fig. 4C), while GSK-3 β phosphorylation was not affected (Fig. 4C). However, GSK-3 α phosphorylation continued to increase, even after inositol levels were restored (Figs. 4B and 4C), suggesting that inhibition of inositol synthesis, but not inositol depletion, is responsible for inactivation of GSK-3 α .

DISCUSSION

Despite the importance of inositol, there are very few reported studies of the consequences of inhibition of inositol synthesis in human cells. Here, we report that inositol synthesis is essential for proliferation and neurite outgrowth of SK-N-SH human neuroblastoma cells, and that inhibition of inositol synthesis leads to GSK-3 α inactivation.

The interplay between inositol biosynthesis and GSK-3 activity reported in the current study has implications for understanding the therapeutic mechanisms of the mood-stabilizers used to treat bipolar disorder. Lithium and VPA are mood-stabilizers with disparate chemical properties. Interestingly, both drugs have been shown to decrease cellular inositol content by blocking inositol biosynthesis, and both drugs also inhibit GSK-3 activity. Consistent with these findings, two prevailing hypotheses for the therapeutic mechanisms of action of these drugs are inositol depletion (Berridge et al., 1989) and GSK-3 inhibition (Klein and Melton, 1996). While the common effects of lithium and VPA on inositol signaling and GSK-3 highlight the potential interplay between inositol metabolism and GSK-3 regulation, several studies reported different effects of these drugs on inositol signaling and GSK-3 (Eickholt et al., 2005; Jin et al., 2005; Whitworth et al., 1990), which complicates our understanding of the mechanism of action of these drugs. The drug-induced perturbation of inositol signaling and GSK-3 activity may be dependent on cell type and study model, the pattern of treatment, and experimental procedures. A potential link between inositol depletion and GSK-3 inhibition has not been previously tested in human cells. While the inositol-depleting drug lithium leads to increased phosphorylation of GSK-3 β (Beaulieu et al., 2008; Zhang et al., 2003), we showed that VPA causes transient inositol depletion leading to increased phosphorylation of GSK-3 α . Interestingly, during VPA treatment, GSK-3 α phosphorylation continued to increase even after inositol levels were restored, suggesting that inhibition of inositol synthesis, but not inositol depletion, is sufficient to inactivate GSK-3 α . We further report a direct link between inositol synthesis and GSK-3 activity in neuronal cells. Specifically, inositol synthesis inhibited by knocking down *INO1* expression results in GSK-3 α inactivation.

While GSK-3 α phosphorylation was increased in response to inhibition of inositol synthesis, GSK-3 β was not significantly altered. Previous studies have shown that inhibitory phosphorylation and kinase activity of GSK-3 are affected by exposure to lithium and VPA (Beaulieu et al., 2008; Chen et al., 1999; Chen et al., 2006; De Sarno et al., 2002; Kim et al.,

2005; Kim et al., 2013; Klein and Melton, 1996; Lucas and Salinas, 1997; Phiel et al., 2003; Ryves and Harwood, 2001; Zhang et al., 2003). While some studies characterized the inhibitory effects of the drugs on both GSK-3 α and GSK-3 β , most have focused on the effects on GSK-3 β . This is the first demonstration in SK-N-SH cells that a decrease in inositol biosynthesis led to the preferential inactivation of GSK-3 α . Further studies are needed to elucidate the significance of the differential inhibition of GSK-3 α and GSK-3 β , which do not have identical functions.

Inactivation of GSK-3 α in SK-N-SH cells was dependent on the degree of Ino1 knockdown (Fig. 4A). The degree of inactivation of GSK-3 α also correlated with exposure times to VPA (Fig. 4C). While inhibition of inositol synthesis potently increases GSK-3 α phosphorylation, inositol homeostasis is highly maintained, as intracellular inositol levels were restored upon prolonged exposure to VPA (Fig. 4B). A similar VPA effect was observed in our previous study in mice, in which mouse brain inositol was only depleted following acute, but not chronic, VPA administration (Shaltiel et al., 2004). It is likely that turnover of phosphatidylinositol and/or recycling of inositol phosphates are increased to compensate for decreased inositol biosynthesis when subjects are exposed to VPA. However, the mechanism whereby inhibition of inositol synthesis causes inactivation of GSK-3 α activity (by increasing phosphorylation of this kinase) remains unclear. Perturbation of inositol homeostasis resulting from disrupted inositol synthesis may modulate PI3K/AKT signaling. We speculate that inhibition of inositol synthesis activates the synthesis of PI3,4,5P3. The synthesis of PI3,4,5P3 is required for recruiting AKT, and the subsequent activation of AKT on the plasma membrane potently inhibits GSK-3 by phosphorylation (Cantley, 2002; Czech, 2003; Di Paolo and De Camilli, 2006). The inactivation of GSK-3 α in response to inhibition of inositol synthesis suggests that GSK-3 α may have a unique role in regulating cellular activity. GSK-3 α has been shown to regulate amyloid- β production by processing the amyloid precursor protein in cultured cells and in mouse brain, suggesting that GSK-3 α may be a valuable target for the treatment of Alzheimer's disease (Phiel et al., 2003). We speculate that GSK-3 α may regulate proliferation. Therefore, inactivation of GSK-3 α by inhibition of inositol synthesis may lead to decreased proliferation.

Surprisingly, exogenous inositol did not affect proliferation or GSK-3 α phosphorylation, suggesting that exogenous inositol does not regulate inositol synthesis. Indeed, exogenous inositol did not affect inositol biosynthesis or uptake by controlling expression of the inositol biosynthetic gene *INO1* or the genes encoding inositol transporters SMIT1 and HMIT. In contrast, the transcription of *INO1* and the inositol transporter genes in yeast cells is highly regulated in response to exogenous inositol, and inositol synthesis and uptake are modulated by this regulation (Henry et al., 2014; Hirsch and Henry, 1986; Lai et al., 1995; Lai and McGraw, 1994; Loewen et al., 2004). This indicates that neuronal cells have evolved different mechanisms to regulate inositol metabolism. For example, inositol uptake in mammals is regulated by glucose, pH, osmolality, and growth factors (Di Daniel et al., 2009; Fu et al., 2012; Miyakawa et al., 1999; Novak et al., 1999; Olgemoller et al., 1993; Spizz and Pike, 1992; Uldry et al., 2004). While the genes encoding inositol transporters are not transcriptionally regulated in response to exogenous inositol in SK-N-SH cells, the

activity of inositol uptake may be controlled by different mechanisms. Inositol synthesis in yeast is also regulated by the synthesis of inositol pyrophosphates (Ye et al., 2013) and the glycolysis intermediate dihydroxyacetone phosphate (DHAP) (Migaud and Frost, 1996; Shi et al., 2005), and requires GSK-3 (Azab et al., 2007). One or more of these mechanisms identified in yeast may also control inositol biosynthesis in mammalian cells.

Interestingly, the effects of exogenous inositol on intracellular inositol vary with the experimental conditions. When cells were inoculated and continuously grown to about 70% confluence for about 5 days in inositol-deficient media or inositol-containing media, intracellular inositol levels in cells grown in inositol-deficient media were similar to those grown in inositol-containing media. However, intracellular inositol levels were profoundly decreased in cells that were shifted to inositol-deficient media for 5 hours compared to those shifted to inositol-rich media after they reached about 70% confluence in DMEM supplemented with regular serum. While intracellular inositol levels were greatly altered in response to an acute manipulation of exogenous inositol, it is curious that those levels are similar in cells grown in media with or without inositol supplementation. We speculate that inositol synthesis is upregulated and/or utilization of inositol for phosphatidylinositol synthesis is decreased in cells grown in inositol-deficient media, thus these cells manifest similar inositol levels and are able to grow similar to cells grown in inositol-containing media.

In summary, we showed that *de novo* inositol synthesis catalyzed by Ino1 is required for proliferation of SK-N-SH cells during inositol-deficient conditions and for GSK-3 α activation. These findings have implications for understanding the therapeutic mechanisms of the mood-stabilizers used for treatment of bipolar disorder.

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Abbreviations

Ino1	inositol-3-phosphate synthase
GSK-3	glycogen synthase kinase-3
DHAP	dihydroxyacetone phosphate
VPA	valproic acid
SMIT1	Na ⁺ /inositol transporter
HMIT	H ⁺ /inositol transporter

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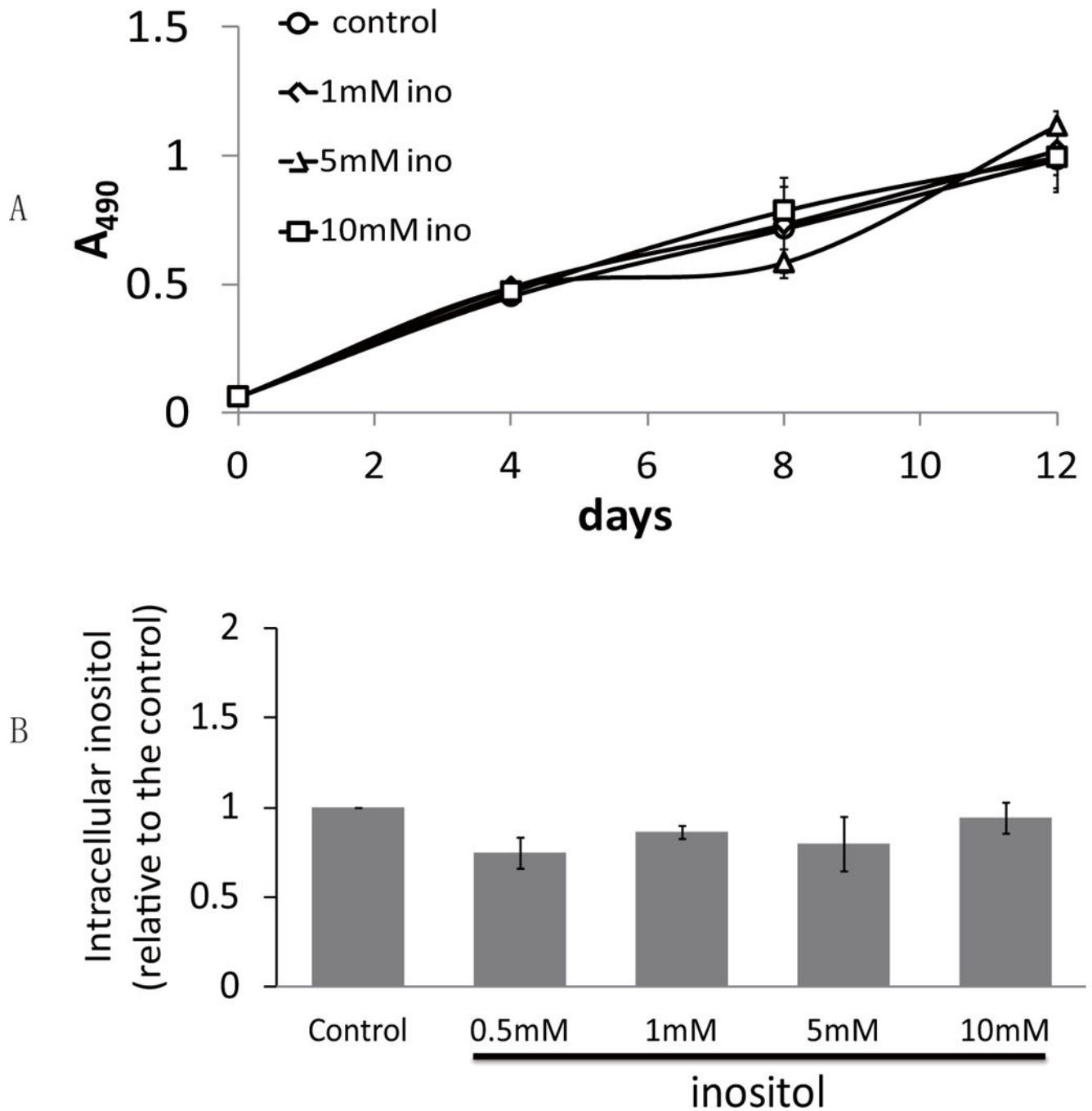


Fig. 1. Exogenous inositol is not essential for cell proliferation or maintaining inositol homeostasis in SK-N-SH cells

(A) SK-N-SH cells were inoculated at a concentration of 5,000 cells per well in 96-well plates at day 0, and cell numbers were estimated by the proliferation assay described under “Materials and Methods.” (B) Intracellular inositol levels were assayed in cells cultured in inositol-deficient media without (control) or with inositol supplement (0.5, 1, 5, 10 mM). The data shown in A and B are the average of at least three experiments \pm S.D, $n = 3$.

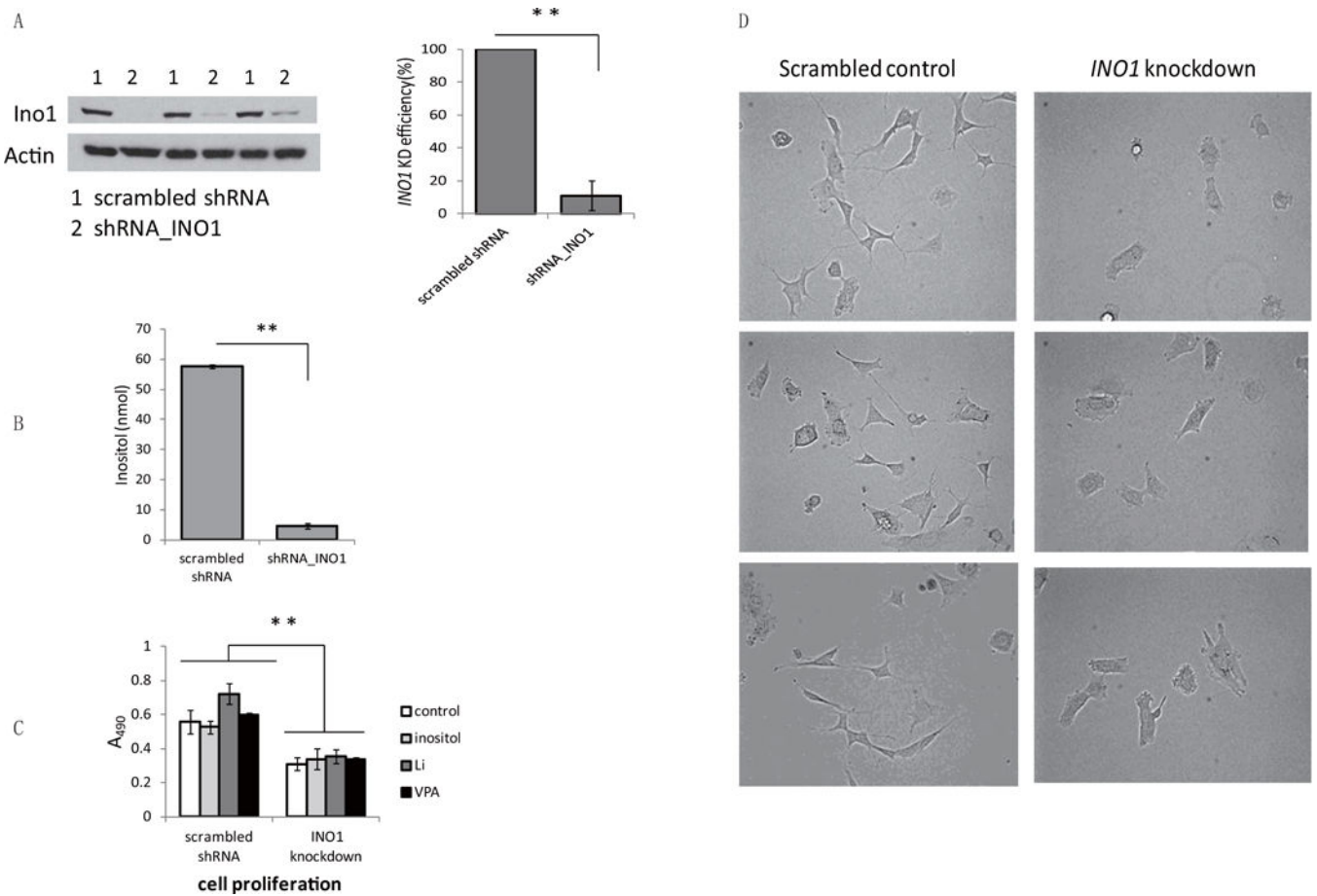


Fig. 2. Inositol biosynthesis is essential for cell proliferation

(A) Western blot analysis of Ino1 protein levels (left) and quantitative analysis of *INO1* knockdown efficiency (right). (B) Inositol levels were measured in control and *INO1* knockdown cells cultured in DMEM supplemented with 10% serum. (C) Cell proliferation was assayed as described in Fig. 1A, and cell numbers were estimated 4 days after inoculation. (D) Control and *INO1* knockdown cells (about 1×10^6) were plated in 100-mm dishes and photographed at day 2 using a microscope at 200 X magnification. The data in A, B, and C are presented as the mean \pm S.D, n=3, and $**p < 0.01$.

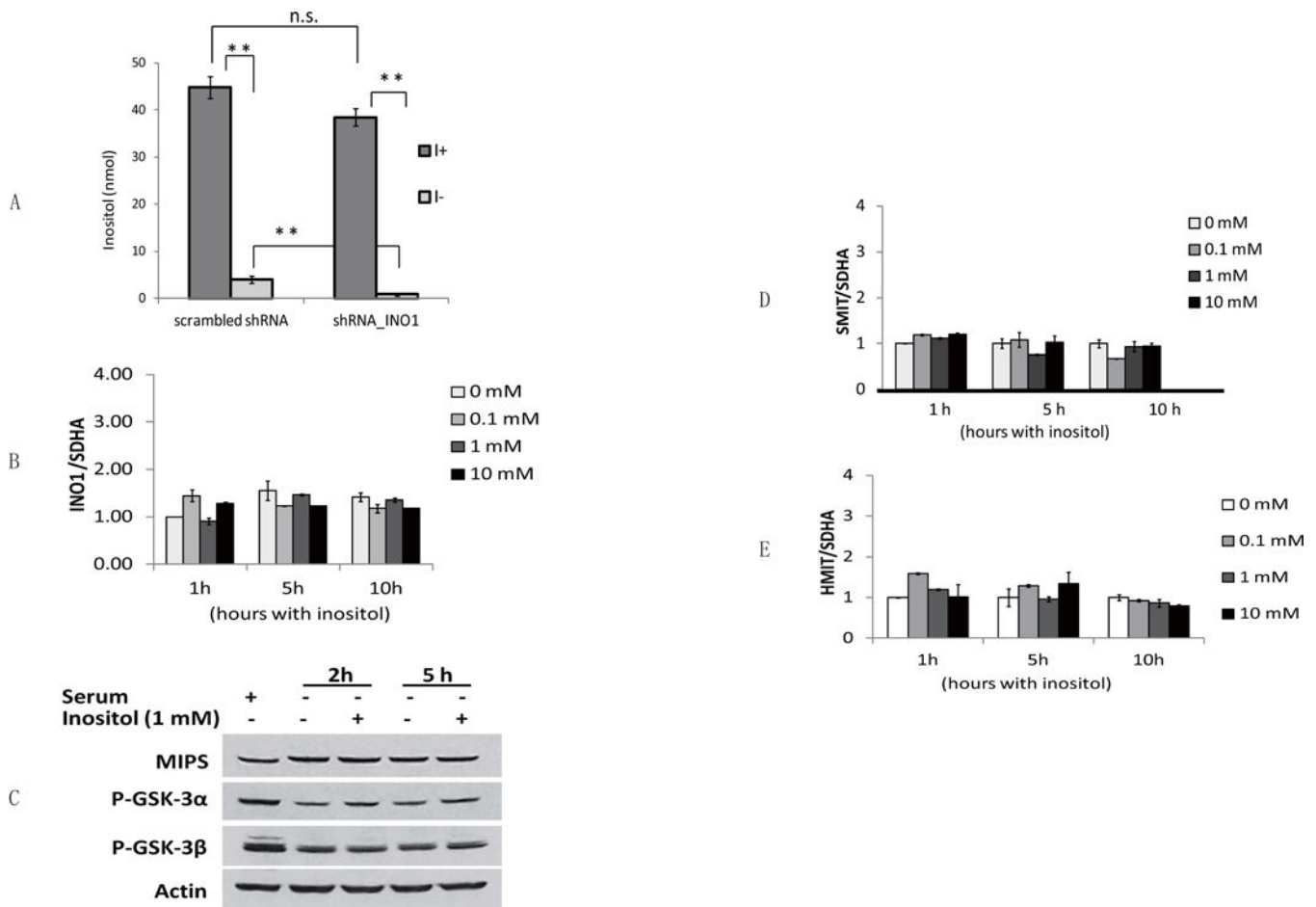


Fig. 3. Exogenous inositol does not regulate transcription of the genes for inositol biosynthesis or uptake

(A) Inositol levels were measured in control or *INO1* knockdown cells in inositol-rich (I+) and inositol-deficient (I-) media. Cells were cultured in DMEM with 10% serum to reach 70% confluence, washed twice with PBS, and replenished with media as indicated. The data are presented as the mean \pm S.D, n=3, and **p<0.01. (B) mRNA levels of *INO1* were measured in SK-N-SH cells incubated in the presence of inositol (0, 0.1, 1, 10 mM) for the indicated times (1, 5, and 10 hours) after growth in inositol-deficient media. Values were normalized to the internal control SDHA (succinate dehydrogenase complex, subunit A). *INO1* mRNA levels normalized to SDHA were represented as fold change relative to cells exposed to 0 mM inositol for 1 hour. (C) Western blot analysis of Ino1 protein levels. SK-N-SH cells were cultured to reach about 70% confluence and then were incubated with either serum or inositol for indicated times. Cells were harvested and lysed for Western blot analysis as described under "Materials and Methods." Actin was used as the loading control. The figure is representative of experiments in triplicate. (D) mRNA levels of Na⁺/inositol transporter SMIT1 and (E) H⁺/inositol transporter HMIT were measured as described above. The data in B, D, and E are presented as the mean \pm S.D, n = 3.

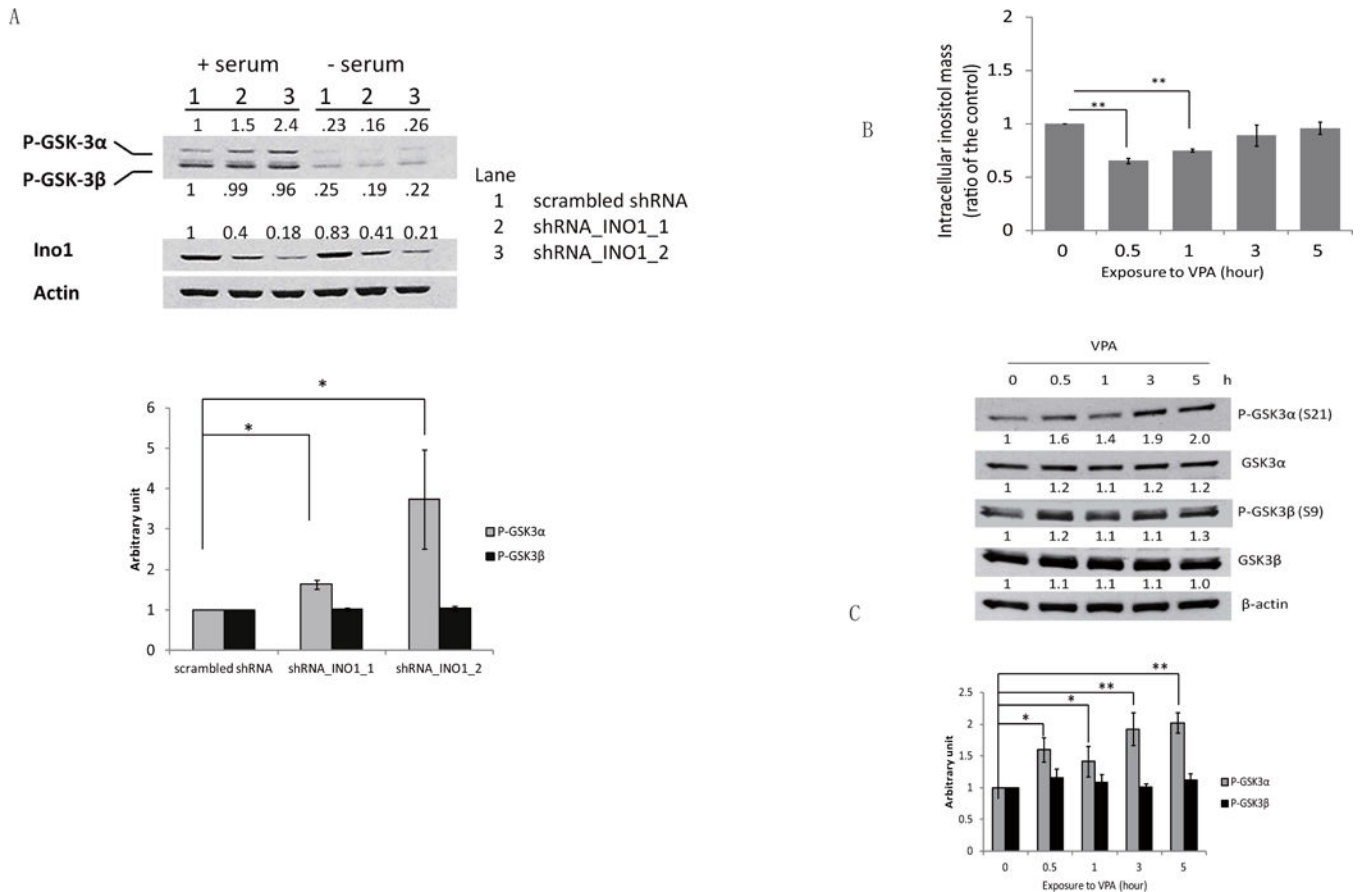


Fig. 4. *INO1* knockdown and VPA treatment lead to inactivation of GSK3 α

(A) Western blot analysis and quantification of Ino1 and inhibitory phosphorylation levels of GSK-3 α (Ser21) and GSK-3 β (Ser9). Actin was used as the loading control. Scrambled control and Ino1 knockdown (shRNA_INO1_1 and shRNA_INO1_2) SK-N-SH cells were cultured to about 70% confluence, and cells were refreshed with media containing 10% serum (+serum) or no serum (-serum) for 4 hours. Cells were harvested and lysed for Western blot analysis as described under “Materials and Methods.” The figure is representative of experiments in triplicate. The quantification data are presented as the mean \pm S.D, $n=3$, and $*p<0.05$. (B) Intracellular inositol levels were measured in SK-N-SH cells after exposure to VPA for the indicated times. The data are presented as the mean \pm S.D, $n=3$, and $**p<0.01$. (C) Western blot analysis and quantification of the protein levels of GSK-3 α (Ser21) and GSK-3 β (Ser9) and total protein levels of GSK-3 α and GSK-3 β . Actin was used as the loading control. The figure is representative of at least three independent experiments. The quantification data are presented as the mean \pm S.D, $n=4$, and $*p<0.05$, $**p<0.01$.

Table 1

Real-time PCR primers used in this study.

Gene	Primers	Sequence (5' to 3')
SDHA	Forward	CGAACGTCTTCAGGTGCTTT
	Reverse	AAGAACATCGGAACTGCGAC
INO1	Forward	CTGCATCGAGAACATCCTCAG
	Reverse	GTTCAACATAGGGTAGGTGGC
SMIT1	Forward	AAGGTGGTGGTTCGAATCTG
	Reverse	CCACAGGATTGTTTGGGTC
HMIT	Forward	CATCTGCAGAATGGTTGCAC
	Reverse	AACTCGCCGAGCTTTAATTG