

Lithium Protection of Phencyclidine-Induced Neurotoxicity in Developing Brain: The Role of Phosphatidylinositol-3 Kinase/Akt and Mitogen-Activated Protein Kinase Kinase/Extracellular Signal-Regulated Kinase Signaling Pathways

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Received October 24, 2007; accepted June 9, 2008

ABSTRACT

Phencyclidine (PCP) and other *N*-methyl-D-aspartate (NMDA) receptor antagonists have been shown to be neurotoxic to developing brains and to result in schizophrenia-like behaviors later in development. Prevention of both effects by antischizophrenic drugs suggests the validity of PCP neurodevelopmental toxicity as a heuristic model of schizophrenia. Lithium is used for the treatment of bipolar and schizoaffective disorders and has recently been shown to have neuroprotective properties. The present study used organotypic corticostriatal slices taken from postnatal day 2 rat pups to investigate the protective effect of lithium and the role of the phosphatidylinositol-3 kinase (PI-3K)/Akt and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathways in PCP-induced cell death. Lithium pretreatment dose-dependently reduced PCP-induced caspase-3 activation and DNA fragmentation in layers II to IV of the cortex. PCP elicited

time-dependent inhibition of the MEK/ERK and PI-3K/Akt pathways, as indicated by dephosphorylation of ERK1/2 and Akt. The proapoptotic factor glycogen synthase kinase (GSK)-3 β was also dephosphorylated at serine 9 and thus activated. Lithium prevented PCP-induced inhibition of the two pathways and activation of GSK-3 β . Furthermore, blocking either PI-3K/Akt or MEK/ERK pathway abolished the protective effect of lithium, whereas inhibiting GSK-3 β activity mimicked the protective effect of lithium. However, no cross-talk between the two pathways was found. Finally, specific GSK-3 β inhibition did not prevent PCP-induced dephosphorylation of Akt and ERK. These data strongly suggest that the protective effect of lithium against PCP-induced neuroapoptosis is mediated through independent stimulation of the PI-3K/Akt and ERK pathways and suppression of GSK-3 β activity.

Phencyclidine and other antagonists of the *N*-methyl-D-aspartate (NMDA) receptor-ion channel complex such as dizolcipine maleate (MK-801) and ketamine have been shown to cause schizophrenia-like symptoms in adult humans (Javitt and Zukin, 1991) and have been used in animals to model this disease (du Bois and Huang, 2007). More recently, it has been shown that rats treated with PCP or ketamine during early postnatal development demonstrated schizo-

phrenia-like behavioral changes during early adolescence or as young adults (Wang et al., 2001; Harris et al., 2003; Fredriksson et al., 2004). The ability of olanzapine to prevent PCP-induced neuroapoptosis and behavioral deficits suggests the usefulness of this model in studying the pathophysiology of schizophrenia (Wang et al., 2001). Determining the precise mechanisms of PCP-induced neurodevelopmental apoptosis may help form appropriate strategies for selecting druggable targets for the treatment of schizophrenia.

During the third trimester in humans and the first three postnatal weeks in rodents, neuronal survival critically depends upon trophic support from neurotrophins, such as brain-derived neurotrophin factor (BDNF) and nerve growth

This work was supported by National Institutes of Health Grant DA-02073 (to K.M.J.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.107.133272.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, 5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine (dizolcipine maleate); PCP, phencyclidine; BDNF, brain-derived neurotrophin factor; NMDAR, *N*-methyl-D-aspartate receptor; ERK, extracellular signal-regulated kinase; PI-3K, phosphatidylinositol-3 kinase; GSK, glycogen synthase kinase; AFC, 7-amino-4-trifluorocumarin; ANOVA, analysis of variance; TdT, deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; LY294002 (LY), 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; AR-A014418 (AR-A), *N*-(4-methoxybenzyl)-*N'*-(5-nitro-1,3-thiazol-2-yl)urea; PD98059 (PD), 2'-amino-3'-methoxyflavone; MEK, mitogen-activated protein kinase kinase; TCN, tricyclic; DIV, day in vitro; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; PBS, phosphate-buffered saline; p-, phosphorylated; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2.5-dione; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; CTRL, control.

factor, as well as neurotransmitters, such as glutamate (Pettmann and Henderson, 1998). It has been postulated that deprivation of glutamatergic trophic support disrupts normal neuronal circuitry formation and may underlie the expression of some mental diseases in later life, including schizophrenia (du Bois and Huang, 2007). The NMDAR is thought to be the principle mediator of glutamate trophic activity in the central nervous system (Balazs et al., 1988). The mechanisms underlying NMDAergic trophic support are not completely understood, although involvement of prosurvival signaling transductions coupled to NMDAR has been proposed. For example, the extracellular signal-regulated kinase (ERK) signaling has been implicated in NMDA-mediated protection against glutamate toxicity in cerebellar granule neurons (Zhu et al., 2005). Furthermore, in developing brain, NMDAR blockade by MK-801 decreased ERK activity, although ERK activation by transgenic synRas only partially prevented MK-801-induced neuronal death in neonatal rats (Hansen et al., 2004), implying the involvement of other pathways.

The phosphatidylinositol-3 kinase (PI-3K)/Akt pathway is another well established prosurvival pathway and has been demonstrated to mediate the prosurvival action of NMDA in cerebellar granule neurons (Zhang et al., 1998). PI-3K/Akt promotes cell survival by phosphorylating and inhibiting proapoptotic proteins such as caspase-9 (Cardone et al., 1998) and the serine/threonine kinase glycogen synthase kinase (GSK)-3 β (Cross et al., 1995). Our laboratory recently demonstrated that PCP, an NMDAR blocker, inhibits the PI-3K/Akt pathway and activates GSK-3 β both in cortical cell culture and in intact neonatal rats (Lei et al., 2008). It is noteworthy that activation of the PI-3K/Akt pathway by enhancing synaptic NMDAR strength prevented PCP-induced neuroapoptosis (Lei et al., 2008). Furthermore, consistent with reports from other laboratories (Takadera and Oh-yashiki, 2004; Takadera et al., 2004), we found that the antiapoptotic property of the PI-3K/Akt pathway is largely mediated by inhibiting the activity of GSK-3 β , because GSK-3 small interfering RNA and lithium prevented PCP-induced neuronal death (Lei et al., 2008).

Lithium is currently used in the treatment of bipolar disorder, as well as schizoaffective disorder, although its therapeutic mechanism is uncertain (Burgess et al., 2001). Lithium has been shown to inhibit GSK-3 β activity through direct or indirect mechanisms (Klein and Melton, 1996; Kirshenboim et al., 2004). Recently, increasing evidence suggests that lithium also protects against apoptosis induced by a variety of insults in cultured neurons, including growth factor withdrawal (Jin et al., 2005), β -amyloid administration (Alvarez et al., 1999), and glutamate treatment (Nonaka et al., 1998). Consequently, several targets other than GSK-3 β have been proposed to account for lithium protection. For example, lithium has been shown to act on ERK, PI-3K, Akt, and phospholipase C (Kang et al., 2003; Pardo et al., 2003; Sasaki et al., 2006). Therefore, it is possible that lithium may prevent PCP-induced cell death in primary neuronal culture through mechanisms in addition to GSK-3 β inhibition.

This study was designed to investigate the potential protective effect of lithium on PCP-induced cell death as well as the underlying mechanisms in organotypic brain slice culture, an *in vitro* model that resembles living tissue *in situ* more closely than primary neuronal culture (Vickers and

Fisher, 2004). We hypothesize that lithium protects against PCP-induced cell death by acting on the PI-3K/Akt and ERK pathways in organotypic culture.

Materials and Methods

Animals. Timed pregnant female Sprague-Dawley rats were obtained on day 14 or 18 of pregnancy from Charles River Laboratories, Inc. (Wilmington, MA). They were housed individually with a regular 12-h light:12-h dark cycle (lights on at 7:00 AM and off at 7:00 PM), with food and water available *ad libitum*. On postnatal day 2.5, the pups were killed by decapitation, and their brains were removed and processed for slice culture as described below. The protocol under which this study was performed was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Reagents. PCP was acquired from the National Institute on Drug Abuse (Rockville, MD) and dissolved in distilled water. Slice culture media including Hanks' balanced salt solution, heat-inactivated horse serum, Opti-MEM, neurobasal medium, and B-27 supplement were purchased from Invitrogen (Carlsbad, CA). D-(+)-Glucose solution (10%), 200 mM L-glutamine, and penicillin/streptomycin solution were purchased from Sigma-Aldrich (St. Louis, MO). 7-Amino-4-trifluorocoumarin (AFC), the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-AFC, and the caspase-3 inhibitor *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone were purchased from MP Biomedicals (Irvine, CA). Primary antibodies against phospho-GSK-3 β (Ser9), phospho-AKT (Ser473), phospho-p44/42 ERK (Thr202/Tyr204), total AKT, total ERK, and total GSK-3 were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Mouse monoclonal anti-actin antibody and horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Millipore Bioscience Research Reagents (Temecula, CA). Deoxynucleotidyl transferase (TdT) and biotin-16-dUTP were purchased from Roche Diagnostics (Indianapolis, IN). ABC Elite kit and Vector SG peroxidase substrate were from Vector Laboratories (Burlingame, CA). LY294002 (PI-3K inhibitor), AR-A014418 (GSK-3 β inhibitor), and PD98059 (MEK inhibitor) were purchased from Calbiochem/EMD Biosciences (San Diego, CA). Akt inhibitor triciribine (TCN) (Yang et al., 2004) was a generous gift from the laboratory of Dr. Xiaodong Cheng (University of Texas Medical Branch, Galveston, TX). Lithium chloride (LiCl) was purchased from Sigma-Aldrich.

Drug Treatments. All experiments with brain slice cultures were done on day *in vitro* (DIV) 10. For experiments using PCP only, 3 μ M PCP was added to the medium at indicated time points before sampling. For experiments using LiCl or AR-A014418 (AR-A), drugs were added either 30 min (LiCl) or 1 h (AR-A) before 3 μ M PCP and left in the medium with PCP until sampling. For experiments using PI-3K, Akt, or ERK inhibitors, slices were preincubated with individual kinase inhibitors for an hour before 10 mM LiCl treatment and then challenged with 3 μ M PCP 30 min later without drug removal.

Organotypic Slice Culture. This study used cultures of organotypic brain slices, an *in vitro* model that conserves the biologically relevant structural and functional features of *in vivo* tissues (Vickers and Fisher, 2004), while also allowing manipulation of drugs that can not easily gain access to the brain *in vivo*. Corticostriatal slice cultures were prepared as prescribed previously (Wang and Johnson, 2007). In brief, 2-day-old rat pups were sacrificed by decapitation. The brains were removed quickly and cut into 400- μ m-thick coronal sections under sterile conditions. Three adjacent frontal corticostriatal slices with morphology comparable to sections between A5.3 and A6.8 mm in postnatal day 10 rats (Sherwood and Timiras, 1970) were placed and cultured in inserts with a porous and translucent membranes (Culture Plate Insert; Millipore Corporation, Billerica, MA) at the interface between medium and a CO₂-enriched atmo-

sphere. The initial culture medium was a mixture of 25% inactivated horse serum, 25% Hanks' balanced salt solution, and 50% Opti-MEM, supplemented with 25 mM D-glucose and 1% penicillin/streptomycin. On DIV 3, the medium was switched to serum-free neurobasal medium supplemented with 25 mM D-glucose, 1 mM glutamine, 2% B-27, and 1% penicillin/streptomycin. The medium was changed twice a week thereafter. Slices were ready for experimental conduction on DIV 10.

Caspase-3 Activity Assay. Slice samples used for caspase-3 activity assay were always collected 12 h after PCP treatment. Caspase-3 activity was measured as described previously (Wang and Johnson, 2007). In brief, slices were sonicated in ice-cold lysis buffer containing 25 mM HEPES, pH 7.4, 5 mM MgCl₂, 1.5 mM EDTA, 1.0 mM EGTA, 1 mM dithiothreitol, 0.1% Triton X-100, and 1% protease inhibitor cocktail. After sitting on ice for 15 min, sonicates were centrifuged at 13,000g for 5 min at 4°C. The supernatants were then collected for measurement of caspase-3 activity. Protein level of the samples was measured by using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). To measure the enzyme activity, each sample was prepared in two parallel sets. One set consisted of an equal volume of supernatant and assay buffer (100 mM HEPES, pH 7.4, 2 mM dithiothreitol, 0.1% CHAPS, and 1% sucrose). The other set was a mix of equal volume of the same supernatant and assay buffer containing the selective caspase-3 inhibitor *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone at 0.5 μ M. After incubation at room temperature for 15 min, the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-AFC (25 μ M), was added, and the samples were then incubated at 37°C for 60 min. Fluorescence resulting from cleavage of the substrate was monitored using a microplate fluorometer (Fluoroskan Ascent; Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 405 and 510 nm, respectively. AFC was used as a fluorescent standard. Caspase-3 activity was calculated as the difference of enzyme activities in samples incubated without and with caspase-3 inhibitor and then normalized to protein level. Final caspase-3 activity shown in figures was presented as percentage of the control group.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling. Slice samples used for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) were always collected 12 h after PCP treatment. Slices were first rinsed with 0.01 M PBS, pH 7.2, and then fixed with ice-cold 2% paraformaldehyde in 0.1 M PBS, pH 7.2, at room temperature for 1 h. After washing with 0.01 M PBS, pH 7.2, slices were dehydrated and rehydrated in a sequential ethanol series (70, 90, 100%), incubated with pepsin (0.04% in 10 mM HCl) for 15 min, and then endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 min. After washing with PBS and preincubation with TdT reaction buffer (30 mM Tris-HCl, pH 7.2, 140 mM Na-cacodylate, and 1 mM CoCl₂) for 15 min, slices were incubated with 10 nmol/ml biotin-16-dUTP and 200 U/ml TdT in the TdT buffer in a humidified chamber for 2 h at 37°C. Slices were then washed in PBS, incubated with ATP-binding cassette reagents for 60 min, and stained with a filtered mixture of Vector SG peroxidase substrate. For quantification, stained TUNEL-positive cells were counted in a photomicrograph of one microscopic field of each slice using the computer-based image analysis program SimplePCI (Compix Inc. Imaging Systems, Cranberry Township, PA).

Western Blot Analysis. Slices were collected and sonicated in 0.3 ml of lysis buffer (50 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, and 0.5% Nonidet P-40) containing the protease inhibitors cocktail and 10 μ l/ml phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail I and II (Sigma-Aldrich). After sitting on ice for 15 min, sonicates were centrifuged at 10,000g for 10 min. The supernatants were collected for immediate use or stored at -80°C. After measuring protein concentration, equal amounts of protein (30 μ g) were loaded and separated on 10% SDS-polyacrylamide gels with a Tris-glycine running buffer system and then transferred to a polyvinylidene difluoride membrane. After blocking in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20, the

membranes were incubated overnight at 4°C with specific antibodies [anti-phospho-Ser9-GSK-3 β (1:2000), anti-phospho-Ser473-Akt (1:2000), and anti-phospho-Thr202/Tyr204 ERK1/2 (1:2000)] and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature. After extensive washes in Tris-buffered saline containing 0.1% Tween 20, blots were visualized by enhanced chemiluminescence (ECL Plus; GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's instructions. To ensure equivalent protein loading, membranes were stripped and reprobed with anti-actin antibody, which was subsequently used to normalize the Western analyses.

Total protein levels of ERK, Akt, and GSK-3 β in the same samples were determined in another membrane. Membranes were incubated with anti-ERK, anti-Akt, or anti-GSK-3 β antibodies at a dilution of 1:1000 for 2 h at room temperature and then with secondary antibodies for 1 h. Blots were developed as indicated above. Equivalent protein loading was determined by stripping and reprobing the membrane with anti-actin antibody.

The bands were scanned and densitometrically analyzed using an automatic image analysis system (Alpha Innotech, San Leandro, CA). All target proteins were quantified by normalizing to β -actin reprobed on the same membrane and then calculated as percentage of control group.

Statistical Analysis. All experimental data are presented as mean \pm S.E. One-way analysis of variance (ANOVA) or two-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons, was used to determine differences among more than two groups. Differences were considered significant when $p < 0.05$.

Results

It has been demonstrated previously that PCP causes neuronal apoptosis in developing rat brain (Wang et al., 2001; Wang and Johnson, 2007) as well as in primary culture of dissociated neurons (Lei et al., 2008) and in organotypic corticostriatal slices (Wang and Johnson, 2007). PCP and dizolcypine are relatively potent blockers of open NMDA channels. In primary culture of dissociated cortical neurons, PCP-induced neurotoxicity is prevented by 10 μ M NMDA (Lei et al., 2008). This strongly suggests that PCP-induced apoptosis is due to blockade of NMDA receptors. To further support this contention, we compared the potency of MK-801 and PCP as NMDA channel blockers to cause caspase-3 activation in cultured organotypic corticostriatal slices. Because ionization is known to affect NMDA binding potency, it is important to note that the pK_a values for MK-801 and PCP are 8.37 and 8.5, respectively (Dravid et al., 2007). The EC₅₀ values for caspase-3 activation were determined to be 23 ± 3 nM for MK-801 and 426 ± 160 nM for PCP. The corresponding K_i values from published binding experiments are 3.8 and 52 nM (Javitt and Zukin, 1989). The calculated EC₅₀/ K_i values for MK-801 and PCP are 6.5 and 8.2, respectively. The similarity in these values for PCP and MK-801 provides additional support for the contention that the PCP-induced increase in caspase-3 activity is mediated by blockade of the NMDA receptor.

Lithium Chloride Protects Corticostriatal Slices from PCP-Induced Neurotoxicity. Our previous study has demonstrated that staining of caspase-3 immunoreactivity was well correlated with caspase-3 enzymatic activity as well as with TUNEL staining (Wang and Johnson, 2007). Therefore, in the current study we used both caspase-3 activation and DNA fragmentation as detected by TUNEL as two indices of PCP-induced apoptosis. In this study, corticostria-

tal slices were first incubated with a series concentration of lithium (0, 0.3, 1, 3, and 10 mM). Thirty minutes later, 3 μ M PCP was added to the medium without washing lithium out. Twelve hours after PCP treatment, slices were collected for either caspase-3 enzymatic assay or TUNEL staining. As reported previously, PCP caused robust caspase-3 activation and DNA fragmentation (as indicated by positive TUNEL staining) that was primarily restricted to layers II to IV in the cortex (Wang and Johnson, 2007). Lithium pretreatment prevented PCP-induced caspase-3 activation ($F_{6,35} = 23.365$; $p < 0.001$) and DNA fragmentation ($F_{5,30} = 8.671$; $p = 0.001$) in a concentration-dependent manner (Fig. 1). Lithium showed no protective effect at either 0.3 or 1 mM ($p > 0.05$ versus PCP). Lithium (3 mM) partially inhibited PCP-induced caspase-3 activation ($p > 0.05$ versus control; $p > 0.05$ versus PCP) and DNA fragmentation ($p < 0.05$ versus PCP; $p < 0.05$ versus control), whereas 10 mM lithium completely blocked PCP-induced cell death ($p < 0.001$ versus PCP). Lithium (10 mM) did not cause any toxicity to the slice ($p > 0.05$ versus control).

PCP Inhibits the PI-3K/Akt/GSK-3 β and MEK/ERK Pathways. Upon activation, NMDAR allows calcium influx, leading to activation of the PI-3K/Akt/GSK-3 β and MEK/ERK signaling pathways, which have been shown to mediate the prosurvival effect of NMDAR activation (Zhang et al., 1998; Lei et al., 2008). Therefore, we proposed that PCP may cause cell death by inhibiting the two NMDAR-coupled pro-

survival pathways. To test this idea, 3 μ M PCP was added to medium on DIV 10 and incubated with slices for 0 h, 30 min, 2, 4, 8, or 24 h before sampling. Untreated slices were used as controls and are presented as 0 h after PCP in the figure. Slices were then homogenized, and total protein was extracted for Western blot analysis of phosphorylation as well as total level of Akt, GSK-3 β , and ERK1/2, as described under *Materials and Methods*. As shown in Fig. 2, PCP caused time-dependent decrease in phosphorylation of ERK ($F_{6,55} = 13.742$; $p < 0.001$), Akt ($F_{6,69} = 14.298$; $p < 0.001$), and GSK-3 β ($F_{6,57} = 5.898$; $p < 0.001$), without changing total protein levels (data not shown). However, the kinetics of dephosphorylation of the three kinases induced by PCP was different. PCP showed a fast and strong inhibitory effect on the ERK1/2 activity. Thirty minutes after PCP treatment, p-ERK1/2 level already decreased to 49.8% of the control level ($p < 0.05$) and was stable at this level thereafter until 24 h at the end of sampling (38.9% of control; $p < 0.05$). Compared with the action on p-ERK1/2, PCP-induced dephosphorylation of Akt and GSK-3 β was slower. No significant decrease of p-Akt was observed at the time points of 30 min and 1 h ($p > 0.05$). Two hours after PCP treatment, p-Akt decreased to 79.1% of control level ($p < 0.05$) and continued to decrease with time. At 24 h, p-Akt decreased to 56.1% of control ($p < 0.05$). Significant decrease of p-GSK-3 β was observed at 1 h after PCP treatment (74.2% of control; $p < 0.05$) and lasted until 24 h (48% of control; $p < 0.05$).

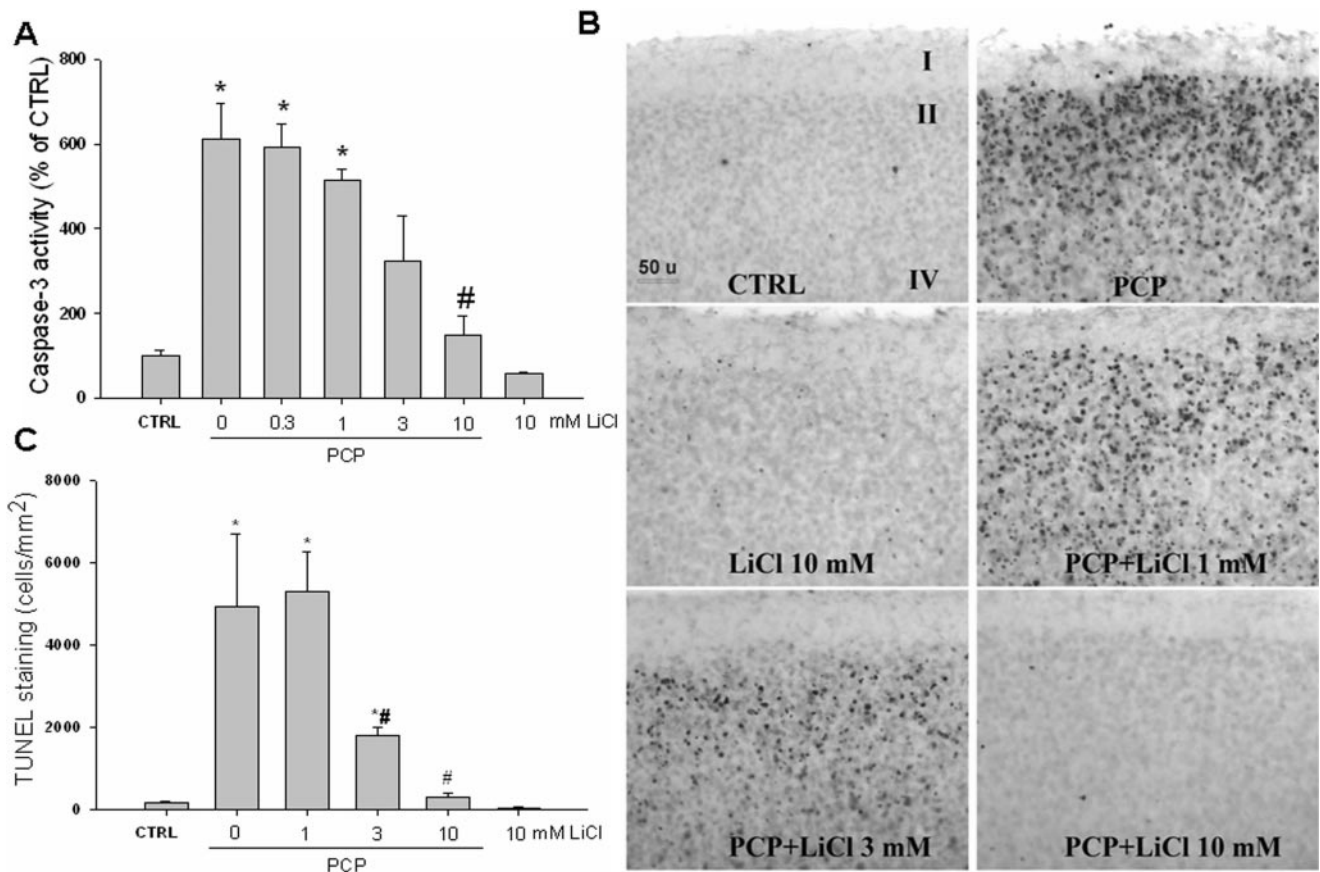


Fig. 1. Effect of lithium on PCP-induced cell death. Corticostriatal slices were pretreated with various concentrations of LiCl on DIV 10 and then challenged with 3 μ M PCP 30 min later without washing LiCl out. Cell death was assessed 12 h after PCP treatment by measuring caspase-3 activity or TUNEL staining in the cortex. Untreated slices were used as control (CTRL). A, lithium blocked PCP-induced caspase-3 activation dose-dependently. B, representative pictures showing TUNEL-positive staining in the cortex. C, quantitative analysis of TUNEL-positive staining. *, $p < 0.05$ versus CTRL; #, $p < 0.05$ versus PCP. One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

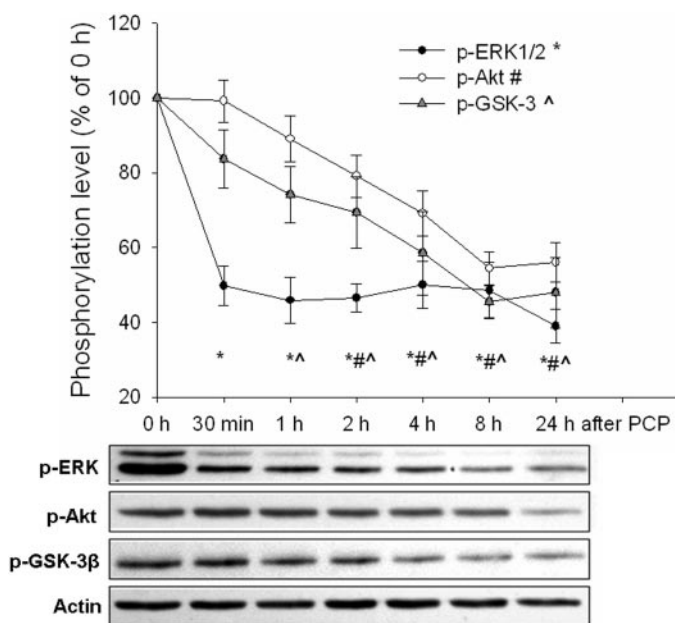


Fig. 2. Temporal effect of PCP on phosphorylation of ERK1/2, Akt, and GSK-3 β in cultured corticostriatal slices. Corticostriatal slices were incubated with 3 μ M PCP for 30 min, 1, 2, 4, 8, or 24 h on DIV 10, and total protein was extracted for Western blotting analysis of phosphorylation of Thr202/Tyr204-ERK1/2, Ser473-AKT, and Ser9-GSK-3 β . Untreated slices were used as control and presented as 0 h after PCP in the figure. Phosphorylation levels of ERK, Akt, and GSK-3 β were normalized to β -actin reprob on the same membrane and then calculated as percentage of control (0 h after PCP). *, p-ERK, $p < 0.05$ versus control (0 h); #, p-Akt, $p < 0.05$ versus control (0 h); ^, p-GSK-3 β , $p < 0.05$ versus control (0 h). One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

These data suggest that PCP may cause cell death through inhibiting the PI-3K/Akt/GSK-3 β and MEK/ERK1/2 pro-survival pathways.

Lithium Prevents PCP-Induced Inhibition of the PI-3K/Akt/GSK-3 β and MEK/ERK Pathways. To determine whether PI-3K/Akt/GSK-3 β and MEK/ERK1/2 pathways were also involved in prevention of PCP-induced cell death by lithium, we first examined the effects of lithium on the kinetics of dephosphorylation of ERK, Akt, and GSK-3 β in-

duced by PCP. Slices were incubated with 3 μ M PCP for 0 h, 30 min, 2 h, or 8 h in the presence or absence of 10 mM LiCl that was added 30 min before PCP. Phosphorylation of ERK1/2, Akt, and GSK-3 β was measured by Western blot analysis. Two-way ANOVA, with lithium treatment and time as two independent factors, revealed that preincubating slices with lithium for 30 min effectively prevented PCP-evoked dephosphorylation of ERK, Akt, GSK-3 β ($p < 0.001$, without lithium versus lithium pretreatment) (Fig. 3). More specifically, lithium pretreatment did not increase basal p-ERK1/2 ($p > 0.05$), but it significantly attenuated the PCP-induced decrease in p-ERK1/2 at the three time points examined (30 min, 2 h, and 8 h) ($p < 0.05$). Preincubating slices with lithium alone for 30 min increased basal p-Akt and p-GSK-3 β by 27.2% ($p < 0.05$) and 16.3% ($p > 0.05$), respectively. This stimulating effect of lithium was suppressed 30 min after addition of PCP to the medium. Furthermore, the level of p-Akt and p-GSK-3 β decreased significantly in the group without lithium at the 2- and 8-h time points ($p < 0.05$), whereas they were still near the basal level in the lithium pretreatment group ($p > 0.05$). At 8 h, lithium pretreatment significantly increased p-Akt and p-GSK-3 β relative to the group without lithium ($p < 0.05$).

The Role of PI-3K/Akt/GSK-3 β Pathway in Lithium-Mediated Protection. As has been shown above, we observed that PCP inhibits, whereas lithium pretreatment stimulates, the PI-3K/Akt pathway. To investigate whether stimulation of this pathway contributes to the neuroprotective effects of lithium, we inhibited the PI-3K/Akt pathway pharmacologically with PI-3K inhibitor LY294002 at 30 μ M or with the Akt inhibitor TCN at 10 μ M. These inhibitors were incubated individually with the slice alone, or followed by 10 mM lithium an hour later. Some slices were challenged with 3 μ M PCP 30 min after lithium treatment. Cell death was assessed 12 h after PCP treatment by measuring caspase-3 activity and TUNEL-positive cells in the cortex. As shown in Figs. 4 and 5, pre-exposure to LY294002 (LY) or TCN abolished the protective effects of lithium ($p < 0.05$ versus PCP + lithium; $p > 0.05$ versus PCP). We also noticed that LY294002 or TCN alone induced significant caspase-3 activation ($p < 0.005$ and $p < 0.001$, respectively, versus

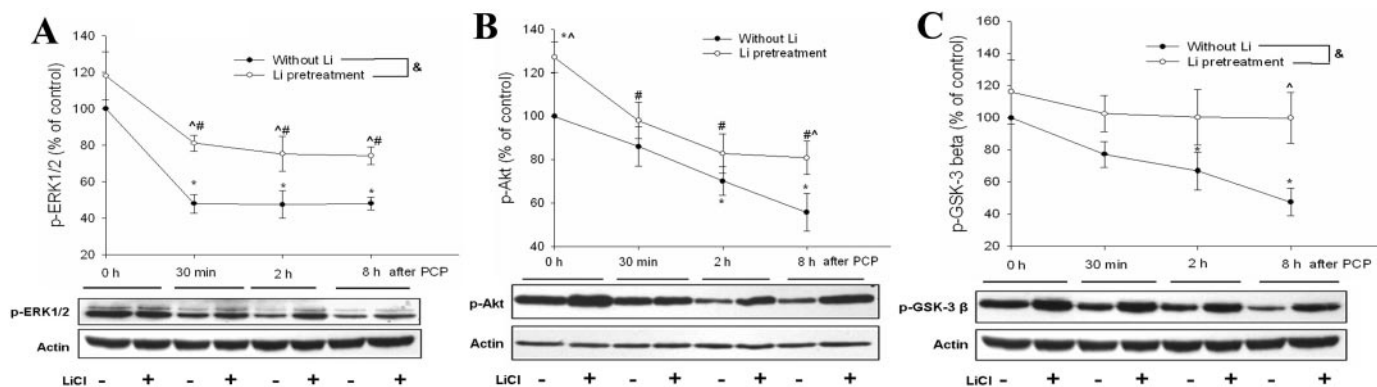


Fig. 3. Effects of lithium on temporal kinetics of PCP-induced dephosphorylation of ERK1/2 (A), Akt (B), and GSK-3 β (C) in cultured corticostriatal slices. On DIV 10, corticostriatal slices, preincubated with or without 10 mM LiCl for 30 min, were incubated with 3 μ M PCP for 0, 30 min, 2 h, or 8 h. Untreated slices were as control and presented as 0 h after PCP in the without lithium group. The group treated with 10 mM LiCl alone for 30 min was presented as 0 h after PCP in the lithium pretreatment group. Phosphorylation levels of ERK (A), Akt (B), and GSK-3 β (C) were normalized to β -actin reprob on the same membrane and presented as percentage of control. &, $p < 0.05$, without lithium versus lithium pretreatment, two-way ANOVA, with time and LiCl treatment as two independent factors. *, $p < 0.05$ versus control; #, $p < 0.05$ versus 0 h after PCP of the lithium pretreatment group; ^, $p < 0.05$, without lithium versus lithium pretreatment at the same time point; Student-Newman-Keuls test for multiple comparisons following two-way ANOVA.

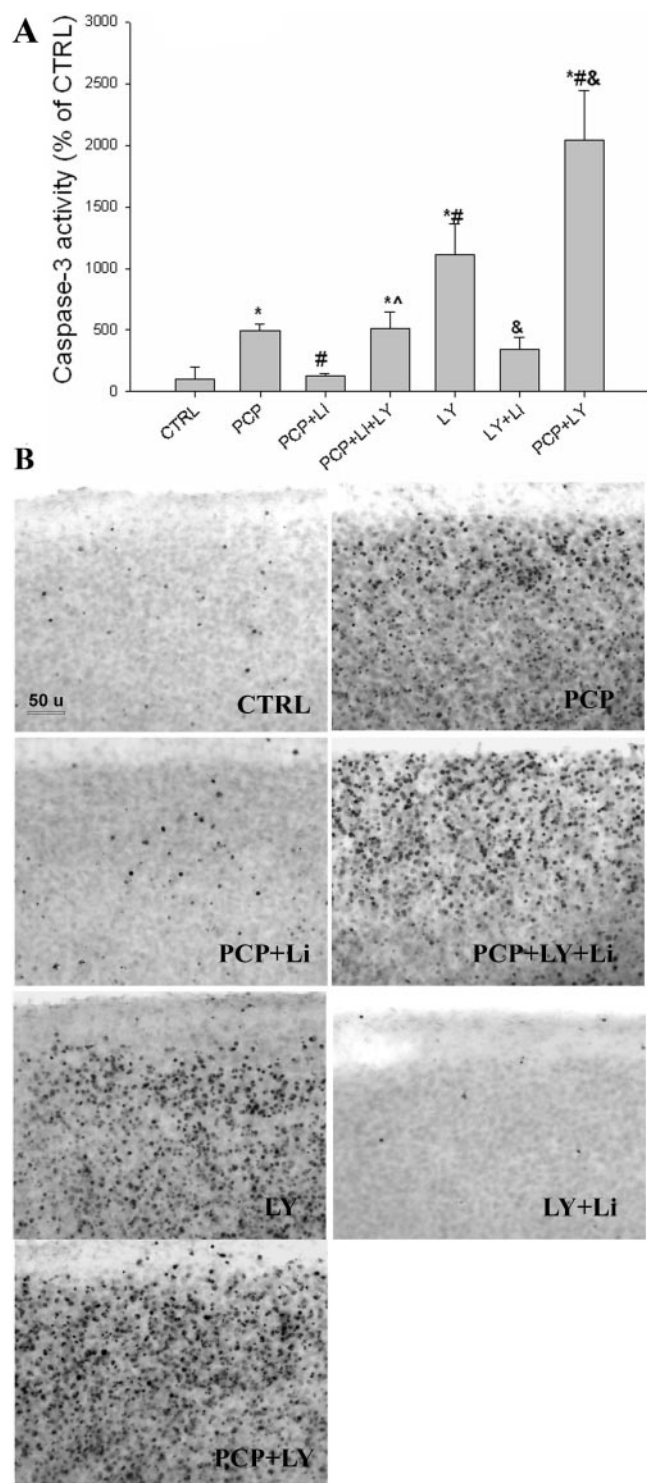


Fig. 4. Inhibition of PI-3K blunts the protective effects of lithium on PCP-induced cell death. Corticostriatal slices were preincubated with 30 μ M LY294002 (LY) for 1 h before 10 mM LiCl treatment. PCP (3 μ M) was added 30 min after LiCl. Cell death was assessed 12 h after PCP treatment by measuring caspase-3 activity or TUNEL staining in the cortex. Untreated slices were used as control (CTRL). A, effect of LY on lithium prevention of caspase-3 activation induced by PCP. B, representative TUNEL-positive staining in the cortex. *, $p < 0.05$ versus control; #, $p < 0.05$ versus PCP alone; ^, $p < 0.05$ versus PCP + lithium; and $p < 0.05$ versus LY alone. One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

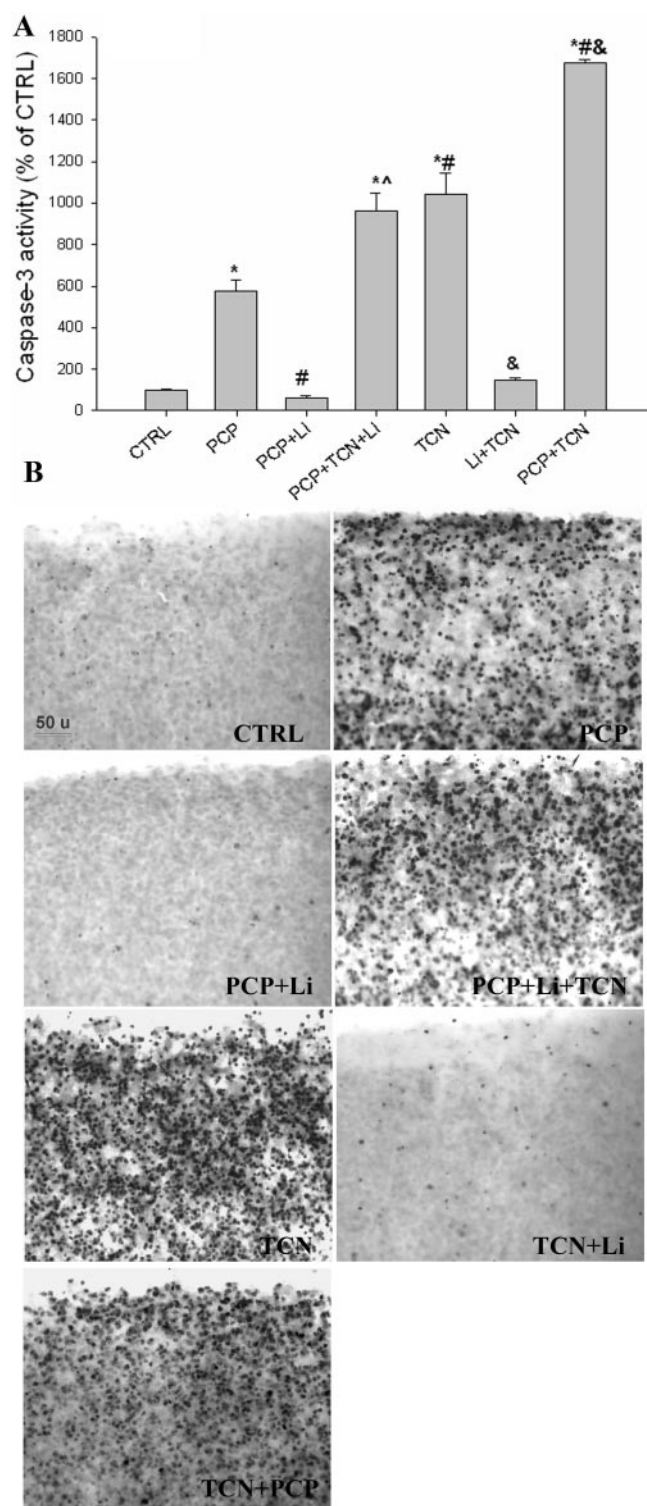


Fig. 5. Akt inhibition diminishes the protective effects of lithium on PCP-induced cell death. Corticostriatal slices were preincubated with Akt inhibitor TCN at 10 μ M for 1 h before 10 mM LiCl treatment. PCP (3 μ M) was added 30 min after LiCl. Cell death was assessed 12 h after PCP treatment by measuring caspase-3 activity or TUNEL staining in the cortex. Untreated slices were used as control (CTRL). A, effect of TCN on lithium prevention of caspase-3 activation induced by PCP. B, representative TUNEL-positive staining in the cortex. *, $p < 0.05$ versus control; #, $p < 0.05$ versus PCP; ^, $p < 0.05$ versus PCP + lithium; and $p < 0.05$ versus TCN alone. One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

control) and caused widely distributed apoptosis in the cortex. It is conceivable that the residual neurotoxicity observed in the inhibitor + PCP + lithium group is due to the intrinsic toxicity of the inhibitors; however, we also observed that the neurotoxicity induced by these inhibitors was largely reversed by lithium ($p < 0.001$, lithium + TCN versus TCN; $p < 0.05$, lithium + LY versus LY), thereby making this possibility unlikely. It is also notable that both PI-3K and Akt inhibitors potentiated neurotoxicity of PCP ($p < 0.05$, PCP + inhibitor versus PCP). Similar results were also observed with another PI-3K inhibitor, wortmannin (data not shown).

Inhibition of PI-3K/Akt leads to dephosphorylation and activation of the proapoptotic factor GSK-3 β (Pap and Cooper, 1998). Accordingly, we observed that PCP caused a significant decrease in p-GSK-3 β (Fig. 2) that was prevented by lithium pretreatment (Fig. 3). To further investigate the role of GSK-3 β , we used a selective GSK-3 β inhibitor, AR-A014418 (Bhat et al., 2003). Slices were preincubated with the indicated concentrations of AR-A014418 for an hour before exposure to 3 μ M PCP. It was found that AR-A014418 inhibited PCP-induced caspase-3 activation ($F_{5, 24} = 30.449$; $p < 0.001$) and DNA fragmentation as measured by TUNEL in the cortex in a dose-dependent manner (Fig. 6). Another specific GSK-3 β inhibitor, SB216763, was also found to protect slices from PCP-induced cell death (data not shown). Taken together, these data indicate that activation of the PI-3K/Akt pathway and the resulting inhibition of GSK-3 β play critical roles in lithium protection of PCP neurotoxicity.

The Role of MEK/ERK Pathway in Lithium-Mediated Protection. The MEK/ERK pathway is another possible signaling transducer involved in the trophic action of NMDAR activity in developing brain (Zhu et al., 2005). We have demonstrated that PCP inhibits ERK1/2 activity and lithium prevents the inhibition (Figs. 2 and 3). To investigate the contribution of MEK/ERK1/2 pathway activation to the protective effect of lithium, we used an MEK inhibitor, PD98059, to block ERK activation pharmacologically. Slices were treated with 30 μ M PD98059 alone or followed by 10 mM lithium an hour later and then challenged with 3 μ M PCP. Cell death was assessed 12 h after PCP treatment. Unlike LY294002 or TCN, PD98059 alone did not cause significant caspase-3 activation ($p > 0.05$ versus control) or DNA fragmentation in the cortex (Fig. 7). However, the neuroprotection afforded by lithium against the PCP insult was significantly reduced by PD98059 exposure ($p < 0.001$ versus PCP + lithium) (Fig. 7). This finding was confirmed with another MEK inhibitor, U0126 (data not shown). These data indicate that the MEK/ERK signaling pathway is also involved in protection by lithium against PCP neurotoxicity.

Lack of Cross-Talk between the PI-3K/Akt/GSK-3 β and MEK/ERK Pathways. The preceding data have shown that both the PI-3K/Akt/GSK-3 β and MEK/ERK pathways were affected by PCP application and were involved simultaneously in the protective effects of lithium against PCP-induced neurotoxicity. Considering that PCP-induced dephosphorylation of ERK1/2 occurred much earlier than that of either Akt or GSK-3 β (Fig. 2), we questioned whether ERK could be involved in either the inhibition of the PI-3K/Akt pathway by PCP or its activation by lithium pretreatment. To answer this question, we determined the phosphorylation levels of ERK, Akt, and GSK-3 β in slices preincubated with

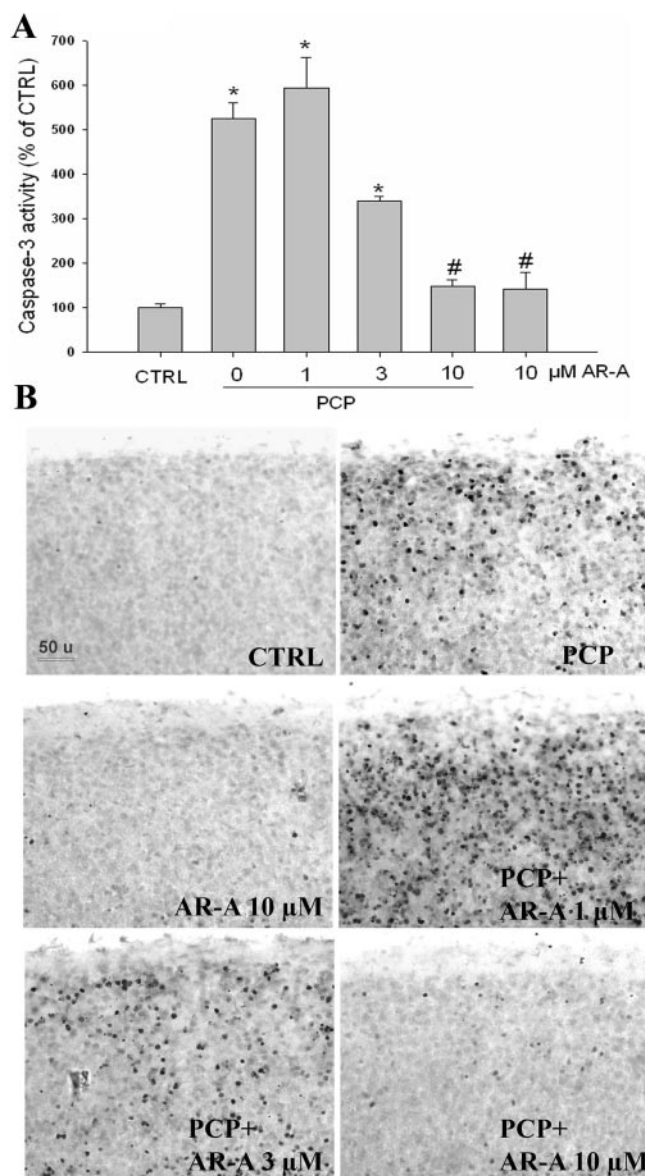


Fig. 6. Effect of the GSK-3 β inhibitor AR-A014418 on PCP-induced cell death. Corticostriatal slices were pretreated with indicated concentrations of AR-A014418 on DIV 10 for 1 h and then challenged with 3 μ M PCP. Cell death was assessed 12 h after PCP treatment by measuring caspase-3 activity or TUNEL staining in the cortex. Untreated slices were used as CTRL. A, AR-A blocked PCP-induced caspase-3 activation. B, representative TUNEL-positive staining in the cortex. *, $p < 0.05$ versus control; #, $p < 0.05$ versus PCP. One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

either PI-3K or MEK inhibitors in the presence or absence of lithium and/or PCP. As expected, the PI-3K inhibitor LY294002 specifically decreased the basal level of p-Akt and p-GSK-3 β ($p < 0.001$ versus control), but it had no effect on p-ERK1/2, whereas the MEK/ERK inhibitor PD98059 selectively inhibited basal activity of ERK1/2. Furthermore, although lithium prevented PCP-induced dephosphorylation of ERK1/2, Akt, and GSK-3 β , LY294002 pre-exposure abolished the preventive effect of lithium on p-Akt and p-GSK-3 β ($p < 0.001$ versus PCP + lithium), but not that on p-ERK ($p > 0.05$ versus PCP + lithium). Analogously, PD98059 blocked the preventive effect of lithium on p-ERK1/2 ($p < 0.001$ versus PCP + lithium), but not that on p-Akt or p-GSK-3 β

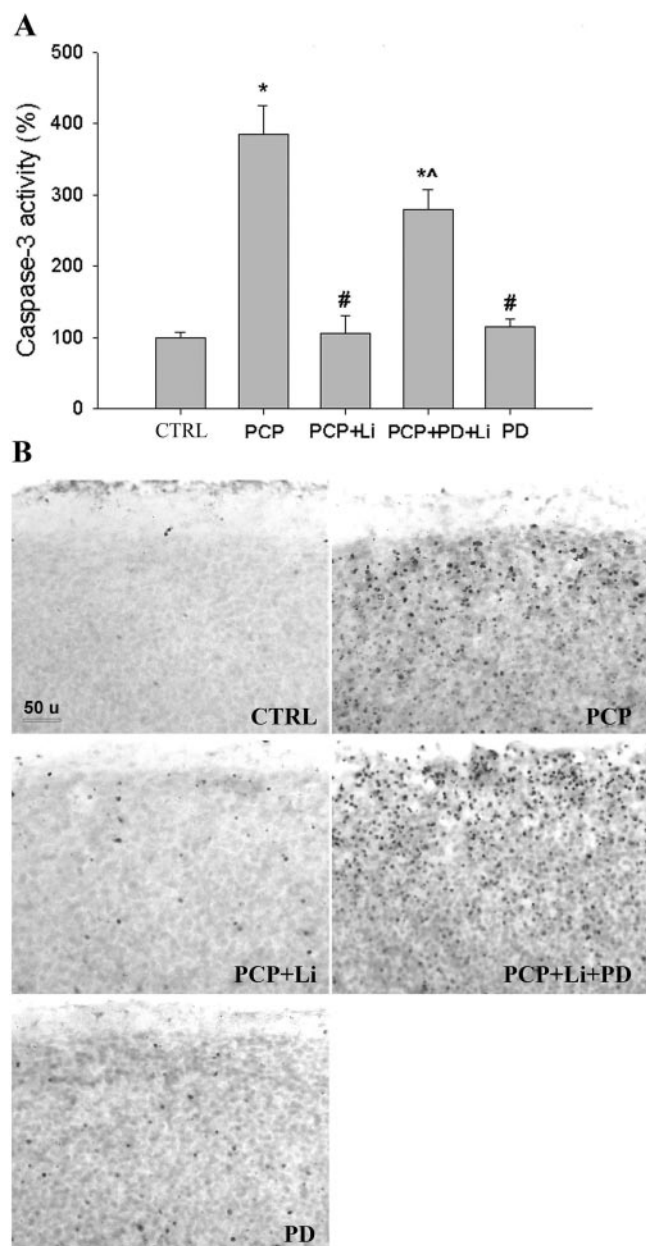


Fig. 7. MEK inhibition attenuates the protective effects of lithium against PCP-induced cell death. Corticostriatal slices were preincubated with 30 μ M PD98059 (PD) for 1 h before 10 mM LiCl treatment. PCP (3 μ M) was added 30 min after LiCl. Cell death was assessed 12 h after PCP treatment by measuring caspase-3 activity or TUNEL staining in the cortex. Untreated slices were used as CTRL. A, effect of PD98059 on lithium inhibition of caspase-3 activation induced by PCP. B, representative TUNEL-positive staining in the cortex. *, $p < 0.05$ versus control; #, $p < 0.05$ versus PCP; ^, $p < 0.05$ versus PCP + lithium. One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

(Fig. 8). These data strongly suggest that although both the MEK/ERK and PI-3K/Akt pathways are involved in lithium protection, there is no cross-talk between their activation, at least at the level of Akt and ERK.

The Role of GSK-3 β in Lithium Protection. Lithium is a well known GSK-3 β inhibitor (Klein and Melton, 1996). In the above-mentioned study, we observed that the PI-3K inhibitor LY294002 blocked prevention of PCP-induced dephosphorylation of GSK-3 β by lithium, suggesting that lith-

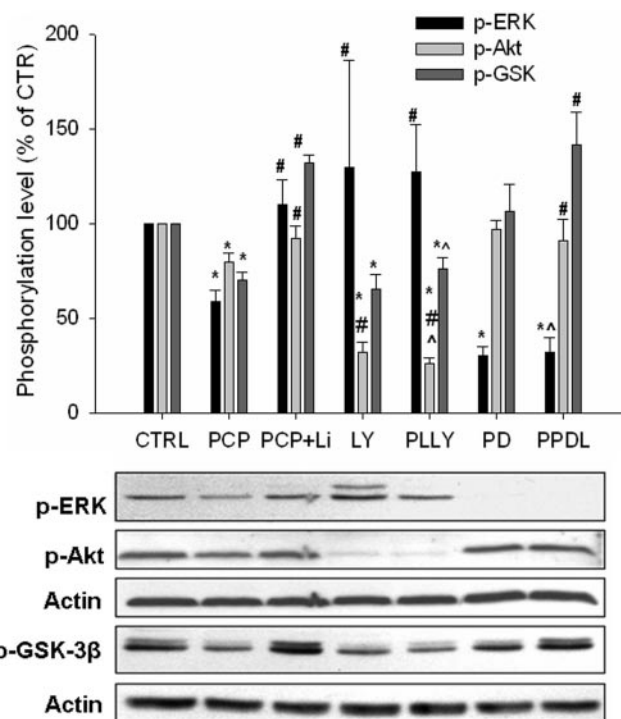


Fig. 8. Relationship between MEK/ERK and PI-3K/Akt/GSK-3 β pathways. Corticostriatal slices were pretreated with PI-3K inhibitor LY or ERK inhibitor PD for 1 h before they were incubated with 10 mM LiCl and then challenged with 3 μ M PCP 30 min later. Slices were collected 8 h after PCP treatment for Western blot analysis of phosphorylation of Thr202/Tyr204-ERK1/2, Ser473-Akt, and Ser9-GSK-3 β . Untreated slices were used as CTRL. Phosphorylation levels of ERK, Akt, and GSK-3 β were normalized to β -actin reprobated on the same membrane and calculated as percentage of the control. PLLY, PCP + lithium + LY; PPDL, PCP + PD + lithium. *, $p < 0.05$ versus control; #, $p < 0.05$ versus PCP; ^, $p < 0.05$ versus PCP + lithium. One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

ium may inhibit GSK-3 β activity indirectly in this model. To further investigate the role of GSK-3 β in protection by lithium against the PCP insult, we measured the activity of Akt and ERK in slices pretreated with the specific GSK-3 β inhibitor AR-A014418. Figure 9 shows that AR-A014418 alone had no effect on the basal phosphorylation level of Akt ($p > 0.01$ versus control) or ERK ($p > 0.1$ versus control). Moreover, although AR-A014418 blocked PCP-induced cell death (Fig. 6), it did not alter the inhibitory effect of PCP on p-Akt ($p > 0.05$, PCP versus PCP + AR-A) or p-ERK ($p > 0.1$, PCP versus PCP + AR-A) (Fig. 9). These data indicate that GSK-3 β is the critical downstream factor that mediates protective effect of lithium.

Discussion

This study found that preincubation with lithium protected corticostriatal slices from PCP neurotoxicity by a mechanism that prevented caspase-3 activation. Our data strongly suggest that the protective effect of lithium is mediated through stimulation of the PI-3K/Akt and MEK/ERK pathways and inhibition of GSK-3 β indirectly.

The PI-3K/Akt/GSK-3 β pathway has been most convincingly implicated in neuronal survival afforded by neurotrophic factors and by NMDAR in cerebellar granule neurons (Zhang et al., 1998; Xifro et al., 2005). NMDAR may activate PI-3K via Ras activation through calcium influx from the

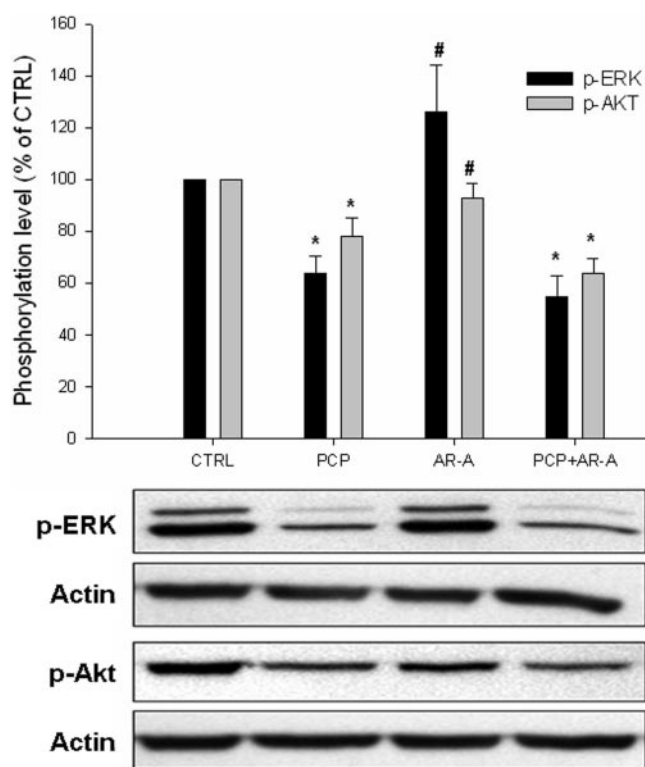


Fig. 9. Effect of the GSK-3 β inhibitor AR-A014418 on PCP-induced dephosphorylation of Akt and ERK. Slices were preincubated with 10 μ M AR-A on DIV 10 for 1 h and then challenged with 3 μ M PCP. Slices were collected 8 h after PCP treatment and used for Western blot analysis of phosphorylation of Thr202/Tyr204-ERK1/2 and Ser473-Akt. Untreated slices were used as CTRL. Phosphorylation levels of ERK and Akt were normalized to β -actin reprobed on the same membrane and calculated as percentage of CTRL. *, $p < 0.05$ versus control. One-way ANOVA, followed by Student-Newman-Keuls test for multiple comparisons.

channel (Hetman and Kharebava, 2006). It has been demonstrated that the NR2B subunit, when phosphorylated at the tyrosine residue near the C terminus, recruits the binding domain of the regulatory p85 subunit of PI-3 kinase via the Scr homology 2 domain, thus leading to activation of PI-3K (Hisatsune et al., 1999). This laboratory showed previously that PCP treatment reduced the activity of Akt, a downstream effector of PI-3K, both in dissociated cortical culture and in intact rat pups (Lei et al., 2008). Furthermore, Lei et al. (2008) demonstrated that inhibitors of either Akt or PI-3K attenuated the protection against PCP neurotoxicity afforded by activation of L-type calcium channels or potentiation of synaptic activity in dissociated cortical culture, indicating that inhibition of PI-3K/Akt signaling may account for PCP-induced cell death.

Here, we show that PCP caused similar inhibition of Akt activity in organotypic culture (Fig. 2). In the presence of lithium, inhibition of Akt by PCP was significantly reduced (Fig. 2). In addition, PI-3K or Akt inhibitors abolished the protective properties of lithium (Figs. 4 and 5), indicating that lithium may protect against the PCP insult through activating the PI-3K/Akt pathway. However, we also observed that blocking the PI-3K/Akt pathway with either LY294002 or TCN caused significant toxicity in the brain slices. It is conceivable that the neurotoxicity observed in the PCP + lithium + inhibitor experimental group may be the result of the intrinsic or residual toxicity of the inhibitor

itself. To exclude this possibility, we included a lithium + inhibitor group in the analysis. We found that lithium attenuated the toxicity of the inhibitors greatly. These data suggest that blockade of the PI-3K/Akt pathway abolishes the protective effect of lithium against PCP. Together, these results strongly support our hypothesis that the PI-3K/Akt pathway is an essential antiapoptotic pathway involved in protection by lithium against PCP-induced cell death.

The Ras/MEK/ERK pathway is another possible player in PCP neurotoxicity and lithium protection in this model. This pathway is activated by calcium influx through NMDA receptors (Krapivinsky et al., 2003), and it has been studied extensively in NMDAR-mediated long-term potentiation (Thomas and Haganir, 2004). Decreased ERK activity has been observed in neonatal rats injected with MK-801 (Hansen et al., 2004). In addition, Hansen et al. (2004) demonstrated that transgenic synRas activation inhibited MK-801-induced developmental neurodegeneration by 40%, indicating a significant role of ERK inhibition in NMDAR blockade-induced neurotoxicity. In accordance with these findings, we also observed a rapid and long-lasting inhibition of ERK1/2 by PCP in cultured corticostriatal slices (Fig. 2), probably by uncoupling NMDAR and ERK signaling via blockade of calcium influx. Lithium pretreatment prevented PCP inhibition of ERK1/2, as well as PCP-induced apoptosis (Figs. 1 and 3). Furthermore, blocking ERK1/2 activity with the MEK inhibitor PD98059 abolished lithium protection (Fig. 7). These data further suggest that the MEK/ERK1/2 pathway plays a role in NMDAR blockade-induced neurotoxicity in the developing brain and that activation of the MEK/ERK1/2 pathway is involved in lithium protection in this model.

The PI-3K/Akt and MEK/ERK pathways play different roles in neuroprotection observed under different conditions. For example, activation of the PI-3K/Akt pathway was shown to be necessary for BDNF protection against serum withdrawal in cortical neurons, but not for BDNF-mediated protection against DNA damage (Hetman et al., 1999). In contrast, activation of the MEK/ERK pathway, but not the PI-3K/Akt pathway, was required for protection both by low concentrations of NMDA against glutamate excitotoxicity in cerebellar granule neurons (Zhu et al., 2005) and for BDNF protection against camptothecin-induced apoptosis in cortical culture (Hetman et al., 1999). Under certain situations, activation of both pathways is required. For example, protection of BDNF against glutamate-induced apoptosis in hippocampal neurons is mediated by both pathways, although cross-talk between the two pathways has been reported (Almeida et al., 2005). In this study, we found that blocking either the PI-3K/Akt or the MEK/ERK pathway attenuated lithium protection, suggesting involvement of both pathways. However, no cross-talk between these pathways was found, because the MEK inhibitor PD98059, only specifically suppressed the stimulating effect of lithium on PCP-induced inhibition of ERK, whereas it had no effect on p-Akt or p-GSK-3 β . Furthermore, the PI-3K inhibitor LY294002 selectively blocked the preventive effect of lithium on PCP-induced dephosphorylation of Akt and GSK-3 β , but not ERK (Fig. 8). These data strongly suggest that although the two pathways equally contributed to lithium protection, their stimulation by lithium is independent. However, it is possible that they may share other common targets downstream.

In this model, our data suggest that GSK-3 β is probably such a downstream common target. Indeed, Takadera and coworkers (Takadera et al., 2004; Takadera and Ohyashiki 2004) have shown that GSK-3 β inhibitors prevented ethanol-, ketamine-, and ifenprodil-induced apoptosis in cortical cell cultures by inactivation of caspase-3. In a recent study, our laboratory reported that inhibition of GSK-3 β by small interfering RNA or other GSK-3 β inhibitors, including lithium, blocked PCP-induced cell death in dissociated cortical cell cultures (Lei et al., 2008). Consistent with these reports, the present study using organotypic corticostriatal slice cultures found that PCP caused time-dependent activation of GSK-3 β by decreasing phosphorylation at serine 9. Lithium and another specific GSK-3 β inhibitor, AR-A014418, also prevented PCP-induced cell death. It is noticeable that unlike lithium, AR-A014418 did not alter PCP inhibition of Akt and ERK activity, implying that neither Akt nor ERK activation is required for the protection afforded through GSK-3 β inhibition. This strongly suggests that GSK-3 β is the critical downstream factor that mediates PCP-induced cell death as well as lithium-mediated protection. In addition, the PI-3K inhibitor LY294002 attenuated the inhibitory effect of lithium on GSK-3 β (Fig. 8), indicating that unlike AR-A014418, lithium inhibited GSK-3 β activity indirectly in this system. It is quite possible that stimulation of the PI-3K/Akt and MEK/ERK pathways by lithium may finally converge on GSK-3 β inhibition to counteract PCP-induced neurotoxicity. However, this hypothesis is confounded by the observation that the MEK inhibitor PD98059 showed no effect on phosphorylation of GSK-3 β at serine 9. It has been reported that ERK activation protects cortical neurons from GSK-3 β activation-induced apoptosis through an unknown mechanism that is independent of serine 9 phosphorylation (Hetman et al., 2002; Habas et al., 2006). Thus, it is possible that in this model, PI-3K/Akt and ERK may be activated by lithium through different mechanisms to suppress the proapoptotic activity of GSK-3 β .

In summary, these data confirmed our previous report of the role of the PI-3K/Akt/GSK-3 β survival pathway in PCP-induced apoptosis in dissociated culture (Lei et al., 2008) and extended these findings by demonstrating that PCP also inhibits the MEK/ERK1/2 survival pathway in corticostriatal slices. Most importantly, the present study demonstrates that the protective effect of lithium against PCP-induced neuroapoptosis in the cortical slice is mediated through co-stimulation of the PI-3K/Akt and MEK/ERK pathways as well as through the indirect suppression of GSK-3 β activity. Finally, the ability of lithium to provide neuroprotection against PCP-induced neurotoxicity lends support to this paradigm as a cellular model of the symptoms of schizophrenia, mania, and other disorders that are well known to be significantly dampened by lithium therapy.

Acknowledgments

We thank Dr. Xiaodong Cheng and Zhenyu Ji for valuable discussions of PI-3K/Akt signaling.

References

- Almeida RD, Manadas BJ, Melo CV, Gomes JR, Mendes CS, Grãos MM, Carvalho RF, Carvalho AP, and Duarte CB (2005) Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death Differ* 12:1329–1343.
- Alvarez G, Muñoz-Montano JR, Satrustegui J, Avila J, Bogóñez E, and Díaz-Nido J

- (1999) Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration. *FEBS Lett* 453:260–264.
- Balázs R, Jørgensen OS, and Hack N (1988) N-Methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. *Neuroscience* 27:437–451.
- Bhat R, Xue Y, Berg S, Hellberg S, Örmö M, Nilsson Y, Radesäter AC, Jerning E, Markgren PO, Borgegård T, et al. (2003) Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. *J Biol Chem* 278:45937–45945.
- Burgess S, Geddes J, Hawton K, Townsend E, Jamison K, and Goodwin G (2001) Lithium for maintenance treatment of mood disorders. *Cochrane Database Syst Rev* 2001:CD003013.
- Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, and Reed JC (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282:1318–1321.
- Cross DA, Alessi DR, Cohen P, and Andjelkovich M (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785–789.
- Dravid SM, Erreger K, Yuan H, Nicholson K, Le P, Lyuboslavsky P, Almonte A, Murray E, Mosely C, Barber J, et al. (2007) Subunit-specific mechanisms and proton sensitivity of NMDA receptor channel block. *J Physiol* 581:107–128.
- du Bois TM and Huang XF (2007) Early brain development disruption from NMDA receptor hypofunction: relevance to schizophrenia. *Brain Res* 53:260–270.
- Fredriksson A, Archer T, Alm H, Gørth T, and Eriksson P (2004) Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration. *Behav Brain Res* 153:367–376.
- Habas A, Kharebava G, Szatmari E, and Hetman M (2006) NMDA neuroprotection against a phosphatidylinositol-3 kinase inhibitor, LY294002 by NR2B-mediated suppression of glycogen synthase kinase-3 β -induced apoptosis. *J Neurochem* 96:335–348.
- Hansen HH, Briem T, Dziętko M, Siffringer M, Voss A, Rzeski W, Zdzisinska B, Thor F, Heumann R, Stepulak A, et al. (2004) Mechanisms leading to disseminated apoptosis following NMDA receptor blockade in the developing rat brain. *Neurobiol Dis* 16:440–453.
- Harris LW, Sharp T, Gartlon J, Jones DN, and Harrison PJ (2003) Long-term behavioural, molecular and morphological effects of neonatal NMDA receptor antagonism. *Eur J Neurosci* 18:1706–1710.
- Hetman M, Hsuan SL, Habas A, Higgins MJ, and Xia Z (2002) ERK1/2 antagonizes glycogen synthase kinase-3 β -induced apoptosis in cortical neurons. *J Biol Chem* 277:49577–49584.
- Hetman M, Kanning K, Cavanaugh JE, and Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem* 274:22569–22580.
- Hetman M and Kharebava G (2006) Survival signaling pathways activated by NMDA receptors. *Curr Top Med Chem* 6:787–799.
- Hisatsune C, Umemori H, Mishina M, and Yamamoto T (1999) Phosphorylation-dependent interaction of the N-methyl-D-aspartate receptor epsilon 2 subunit with phosphatidylinositol 3-kinase. *Genes Cells* 4:657–666.
- Javitt DC and Zukin SR (1989) Biexponential kinetics of [³H]MK-801 binding: evidence for access to closed and open N-methyl-D-aspartate receptor channels. *Mol Pharmacol* 35:387–393.
- Javitt DC and Zukin SR (1991) Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* 148:1301–1308.
- Jin N, Kovács AD, Sui Z, Dewhurst S, and Maggiorini SB (2005) Opposite effects of lithium and valproic acid on trophic factor deprivation-induced glycogen synthase kinase-3 activation, c-Jun expression and neuronal cell death. *Neuropharmacology* 48:576–583.
- Kang HJ, Noh JS, Bae YS, and Gwag BJ (2003) Calcium-dependent prevention of neuronal apoptosis by lithium ion: essential role of phosphoinositide 3-kinase and phospholipase Cgamma. *Mol Pharmacol* 64:228–234.
- Kirshenboim N, Plotkin B, Shlomo SB, Kaidanovich-Beilin O, and Eldar-Finkelman H (2004) Lithium-mediated phosphorylation of glycogen synthase kinase-3 β involves PI3 kinase-dependent activation of protein kinase C- α . *J Mol Neurosci* 24:237–245.
- Klein PS and Melton DA (1996) A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A* 93:8455–8459.
- Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, Ben-Ari Y, Clapham DE, and Medina I (2003) The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. *Neuron* 40:775–784.
- Lei G, Xia Y, and Johnson KM (2008) The role of Akt-GSK-3 β signaling and synaptic strength in phencyclidine-induced neurodegeneration. *Neuropsychopharmacology* 33:1343–1353.
- Nonaka S, Hough CJ, and Chuang DM (1998) 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 U S A* 95:2642–2647.
- Pap M and Cooper GM (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. *J Biol Chem* 273:19929–19932.
- Pardo R, Andreolotti AG, Ramos B, Picatoste F, and Claro E (2003) Opposed effects of lithium on the MEK-ERK pathway in neural cells: inhibition in astrocytes and stimulation in neurons by GSK3 independent mechanisms. *J Neurochem* 87:417–426.
- Pettmann B and Henderson CE (1998) Neuronal cell death. *Neuron* 20:633–647.
- Sasaki T, Han F, Shioda N, Moriguchi S, Kasahara J, Ishiguro K, and Fukunaga K (2006) Lithium-induced activation of Akt and CaM kinase II contributes to its neuroprotective action in a rat microsphere embolism model. *Brain Res* 1108:98–106.
- Sherwood NG and Timiras PS (1970) *A Stereotaxic Atlas of the Developing Rat Brain*. University of California Press, Berkeley, CA.
- Takadera T and Ohyashiki T (2004) Glycogen synthase kinase-3 inhibitors prevent

- caspase-dependent apoptosis induced by ethanol in cultured rat cortical neurons. *Eur J Pharmacol* **499**:239–245.
- Takadera T, Sakamoto Y, and Ohyashiki T (2004) NMDA receptor 2B-selective antagonist ifenprodil-induced apoptosis was prevented by glycogen synthase kinase-3 inhibitors in cultured rat cortical neurons. *Brain Res* **1020**:196–203.
- Thomas GM and Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci* **5**:173–183.
- Vickers AEM and Fisher RL (2004) Organ slices for the evaluation of human drug toxicity. *Chemicobiol Interact* **150**:87–96.
- Wang C, McInnis J, Ross-Sanchez M, Shinnick-Gallagher P, Wiley JL, and Johnson KM (2001) Long-term behavioral and neurodegenerative effects of perinatal phenylcyclidine administration: implications for schizophrenia. *Neuroscience* **107**:535–550.
- Wang CZ and Johnson KM (2007) The role of caspase-3 activation in phenylcyclidine-induced neuronal death in postnatal rats. *Neuropsychopharmacology* **32**:1178–1194.
- Xifro X, Malagelada C, Miñano A, and Rodríguez-Alvarez J (2005) Brief exposure to NMDA produces long-term protection of cerebellar granule cells from apoptosis. *Eur J Neurosci* **21**:827–840.
- Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI, Hamilton AD, Polokoff M, Nicosia SV, Herlyn M, et al. (2004) Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res* **64**:4394–4399.
- Zhang FX, Rubin R, and Rooney TA (1998) N-Methyl-D-aspartate inhibits apoptosis through activation of phosphatidylinositol 3-kinase in cerebellar granule neurons. A role for insulin receptor substrate-1 in the neurotrophic action of n-methyl-D-aspartate and its inhibition by ethanol. *J Biol Chem* **273**:26596–26602.
- Zhu D, Wu X, Strauss KI, Lipsky RH, Qureshi Z, Terhakopian A, Novelli A, Banaudha K, and Marini AM (2005) N-Methyl-D-aspartate and TrkB receptors protect neurons against glutamate excitotoxicity through an extracellular signal-regulated kinase pathway. *J Neurosci Res* **80**:104–113.

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