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The Role of Akt-GSK-3β Signaling and Synaptic Strength in Phencyclidine-Induced Neurodegeneration

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N-methyl-D-aspartate (NMDA) receptor antagonists such as phencyclidine (PCP) can induce positive and negative symptoms of schizophrenia in humans and related effects in rodents. PCP treatment of developing rats induces apoptotic neurodegeneration and behavioral deficits later in life that mimic some symptoms of schizophrenia. The precise mechanism of PCP-induced neural degeneration is unknown. This study used selective antagonists, siRNA, and Western analysis to investigate the role of the Akt-glycogen synthase kinase-3β (GSK-3β) pathway in PCP-induced neuronal apoptosis in both neuronal culture and postnatal day 7 rats. PCP administration *in vivo* and *in vitro* reduced the phosphorylation of Akt^{Ser427} and GSK-3β^{Ser9}, decreasing Akt activity and increasing GSK-3β activity. The alteration of Akt-GSK-3β signaling parallels the temporal profile of caspase-3 activation by PCP. Reducing GSK-3β activity by application of selective inhibitors or depletion of GSK-3β by siRNA attenuates caspase-3 activity and blocks PCP-induced neurotoxicity. Moreover, increasing synaptic strength by either activation of L-type calcium channels with BAY K8644 or potentiation of synaptic NMDA receptors with either a low concentration of NMDA or bicuculline plus 4-aminopyridine completely blocks PCP-induced cell death by increasing Akt phosphorylation. These neuroprotective effects are associated with activation of phosphoinositide-3-kinase-Akt signaling, and to a lesser extent, the MAPK signaling pathway. Overall, these data suggest that PCP-induced hypofunction of synaptic NMDA receptors impairs the Akt-GSK-3β cascade, which is necessary for neuronal survival during development, and that interference with this cascade by PCP or natural factors may contribute to neural pathologies, perhaps including schizophrenia.
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INTRODUCTION

Phencyclidine (PCP), like MK801 and ketamine, is a non-competitive, use-dependent *N*-methyl-D-aspartate (NMDA) receptor antagonist or channel blocker (Anis *et al*, 1983). It is well known that acute administration of NMDA antagonists can induce a broad range of antipsychotic-sensitive, schizophrenia-like symptomatology in both humans and rodents, findings that have greatly contributed to a hypoglutamatergic hypothesis of schizophrenia (Farber *et al*, 1995; Olney and Farber, 1995; Jentsch and Roth, 1999; Wang *et al*, 2001; Morris *et al*, 2005; Coyle, 2006). The schizophrenia-like behaviors have been attributed to both the acute vacuolization caused by MK-801 and PCP as well as the non-toxic temporary effects of these drugs. In addition, it has been demonstrated that NMDA antagonists administered during early development in rodents induce neurodegeneration in specific brain regions (Ikonomidou *et al*, 1999; Wang and Johnson, 2005) that is associated with schizophrenia-like alterations in sensorimotor gating and working memory later in life (Wang *et al*, 2001). PCP-induced cortical degeneration is caspase-3 dependent and is also developmentally regulated (Ikonomidou *et al*, 1999; Wang and Johnson, 2005, 2007). Since schizophrenia has been recognized as a developmentally dependent disorder, the demonstration that MK801- and PCP-induced neurodegeneration is developmentally regulated lends credence to the value of this animal model and suggests that understanding the mechanism of neuronal death in this model may provide insights into the pathophysiology of schizophrenia as well as its pharmacotherapy.

It has been demonstrated recently that Akt-glycogen synthase kinase-3β (GSK-3β) signaling may be impaired in schizophrenia. The *Akt1* gene is thought to be a potential susceptibility gene for schizophrenia (Emamian *et al*, 2004; Kalkman, 2006; Bajestan *et al*, 2006). Akt is a serine/threonine protein kinase involving diverse cellular processes (Brazil *et al*, 2004). GSK-3β, the predominant brain isoform of GSK-3, is one of the downstream substrates for Akt (Song *et al*, 2002). Physiologically, Akt decreases GSK-3β activity by enhancing GSK-3β phosphorylation, promoting

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cell survival (Cohen and Goedert, 2004; Jope and Roh, 2006). Also, it has been shown that amphetamine-induced impairment of sensorimotor gating and working memory in prefrontal cortex is exaggerated in the Akt1-deficient mouse (Lai *et al*, 2006). Moreover, antipsychotics, such as haloperidol and clozapine, have been reported to enhance Akt-GSK-3β signaling (Kalkman, 2006).

Activation of synaptic NMDA receptors has been reported to enhance neuronal survival signaling cascades, including the phosphoinositide-3-kinase (PI-3K)-Akt pathway (Papadia *et al*, 2005; Soriano *et al*, 2006). PCP blockade prevents calcium influx through physiologically active NMDA receptors, which are typically localized at the synapse. Thus, PCP-induced neurotoxicity could result from blocking signaling through synaptic NMDA receptors. In the current study, we tested the hypotheses that PCP causes neurotoxicity via impairment of the Akt-GSK-3β signaling cascade subsequent to blockade of synaptic NMDA receptors, and further, that this could be prevented by activation of synaptic NMDA receptors and the PI-3K-Akt-GSK-3β pathway.

MATERIALS AND METHODS

Animals and Experimental Paradigms

Timed-pregnant Sprague–Dawley (SD) rats were obtained from Charles River Laboratories Inc. (Wilmington, MA). They were housed individually with a standard 12 h dark/light cycle in a temperature- and humidity-controlled environment with free access to food and water. After parturition, the pups were kept in the same cage with their mother until postnatal day 7 (PN7), and then randomly assigned to either the control or PCP group. PCP (10 mg/kg) or saline was administered subcutaneously. The pups were killed 3, 6, or 9 h afterwards. The cortex, hippocampus, and striatum were dissected with the help of an ice-cold aluminum brain mold and the tissue was quickly immerged in liquid nitrogen for 30 min and stored at -80°C. For Western blots (WBs), frozen tissue was homogenized at 4°C in RIPA buffer (Tris 50 mM, pH 8.0, NaCl 150 mM, EDTA 1 mM, 0.5% Triton-X100, 0.5% deoxycholic acid, 1% phosphatase inhibitor cocktail II, and 1% protease inhibitor cocktail). After centrifugation at 25 000*g* for 30 min, the supernatant was collected and protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL). Procedures were in accordance with the guidelines outlined in the National Institutes of Health *Guide for the Care and Use of the Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of University of Texas Medical Branch.

Primary Neuronal Culture

Embryonic forebrains of E18-19 SD rats were dissociated in cold Hanks solution without Mg^{2+} or Ca^{2+}. Cells were plated at 10×10^5 cells/ml and grown on polylysine (5 mg/ml)-coated multi-well plates in neuorbasal medium containing 0.5 mM L-glutamine, supplemented with 10% B27 at 37°C and 5% CO_2. The culture medium was replaced every 4 days. Experiments were conducted on day *in vitro* (DIV) 14.

Cell Death ELISA

Nucleosomal DNA fragmentation is characteristic of apoptotic nuclei. The presence of fragmented DNA was assessed by measuring DNA associated with nucleosomal histones using a specific two-site ELISA with an antihistone primary antibody and a secondary anti-DNA antibody according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN) as described previously (Wang *et al*, 2005).

MTT Assay

Mitochondrial metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used as an index of mitochondrial viability. MTT solution was added to each well at a final concentration of 5 μg/ml. The cells were incubated for 2 h at 37°C. The medium containing MTT was removed, and DMSO was added to dissolve the intracellular purple formazan metabolite. Color intensity was assessed using a microplate reader at a wavelength of 590 nm.

LDH Assay

The release of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium was used as a marker of plasma membrane integrity and as an index of cell death. Following treatment of cells, the medium was collected and assayed for LDH activity with the Cytotoxicity Detection Kit (LDH) from Roche Applied Science (Indianapolis, IN). The color density was measured using a microplate reader at an absorbance wavelength of 490 nm.

WB Analysis

Cell extracts were prepared by lysis of PBS-washed cells in RIPA buffer. After centrifugation at 20 000 *g*, the supernatant was collected and protein was determined using the BCA™ Protein Assay Kit (Pierce). Antibodies for $pAkt^{Ser473}$, Akt, $pGSK\text{-}3\beta^{Ser9}$, and GSK-3$\beta$ were purchased from Cell Signaling Technology Inc. (Beverly, MA). $pAkt^{Ser473}$ monoclonal antibody does not detect Akt phosphorylated at other sites or related kinases, according to the manufacture. The antibody has been used broadly to detect $pAkt^{Ser473}$ (Jacinto *et al*, 2006). To probe signals on the blots, the concentrations of antibodies used were 1 : 1000 for $pAkt^{Ser473}$ and $pGSK^{Ser9}$, and 1 : 1500 for Akt and GSK-3β, respectively.

siRNA

This experiment was performed following the manufacture's instruction. Briefly, siRNA solutions were prepared by mixing 200 μl of culture medium with the GSK-3 a/β siRNA (Cell Signaling Technology Inc.) stock solution at a final concentration of 0, 50, 100, and 150 nM and the same volume of transfection reagent without siRNA. After incubation for 5 min, the siRNA mixture was added to cells and agitated gently to disperse siRNA evenly. The medium containing siRNA was replaced by fresh medium after 24 h. The inhibition of GSK-3 a/β protein expression was examined by WB 48 h after initial transfection.

Statistical Analyses

Results are presented as mean ± SEM. Significance was determined using a Student's *t*-test or ANOVA with Dunnett's *post hoc* test to determine differences among more than two groups. Differences were considered significant at $P < 0.05$.

RESULTS

Characterization of PCP-Induced Neurotoxicity in Rat Forebrain Neuronal Culture

Neurotoxicity caused by ketamine, PCP, and MK-801 is dependent on development with the most vulnerable time existing in the early postnatal period in rats (Ikonomidou *et al*, 1999; Wang and Johnson, 2005, 2007). Thus, we determined the time frame that neuronal cultures were most vulnerable to PCP. On different DIVs, forebrain cultures were treated for 48 h with PCP (1 μM) and assessed for membrane integrity (LDH release) and mitochondrial viability (metabolism of MTT). We observed (Figure 1a) that this preparation of cultured neurons was sensitive to PCP between DIV13 and DIV18, which is consistent with previous reports (Hwang *et al*, 1999). In subsequent experiments, cultures were used at DIV13 or DIV14. PCP caused loss-of-membrane integrity and mitochondrial viability after 48 h as determined by the LDH and MTT assays, but histone-associated DNA fragmentation was evident as early as 12 h after PCP addition using the Cell Death ELISA assay. PCP-induced toxicity was concentration-dependent with a half maximal effect observed at 65 ± 4.3 nM, which correlates well with its binding affinity of 50–100 nM for NMDA receptors (Johnson and Jones, 1990). As previously reported in corticostriatal slices (Wang and Johnson, 2007), PCP (1 μM) caused activation of caspase-3 within 3 h after PCP treatment, with the peak effect observed at 12 h before decaying at 48 h (Figure 1d). It is possible that the toxic effect of PCP seen *in vivo* could partially result from increased extracellular dopamine (DA) secondary to inhibition of the DA transporter by PCP (Rothman *et al*, 1989; Alagarsamy *et al*, 1997). However, this is very unlikely since this neuronal culture preparation has few, if any, DA neurons. On the other hand, Seeman and Lasaga, 2005 and Seeman *et al*, 2005 reported that PCP has a nanomolar affinity for the high-affinity state of the D2 receptor and thus could have a direct DA-like action on these cells. To exclude this possibility, we preincubated this neuronal culture with 2 μM haloperidol and 10 μM SCH23390 to block DA D2-like and D1-like receptors, respectively. However, neither antagonist had any effect on cell viability measured by MTT metabolism and LDH release (data not shown).

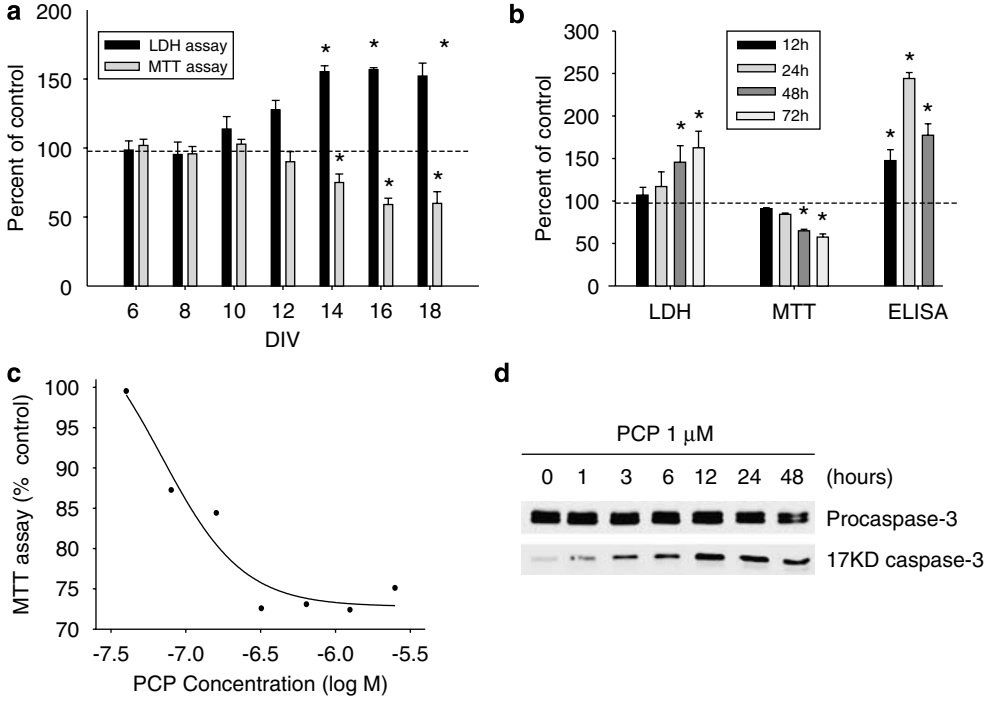

Figure 1 Characterization of PCP induced neurotoxicity in forebrain neuronal cultures. (a) Developmental dependence. Forebrain neurons were cultured for different times (DIV6–DIV16) and exposed to PCP (1 μM) for 48 h. Culture medium (100 μl) was removed from each culture plate well for assay of LDH activity. At this time, 100 μl of MTT solution (5 mg/ml) was added to the plate and incubated for 2 h for assessment of MTT metabolism. (b) Cell death time course. The time course for PCP-induced cell death was determined by measuring LDH release, MTT metabolism, and nucleosomal DNA fragmentation using an ELISA at the indicated times after treatment of DIV14 neurons with 1 μM PCP. (c) PCP concentration dependence. A series of PCP concentrations was applied to cultured neurons (DIV14). Following 48 h exposure, viability was determined using the MTT assay. The dose–response curve was plotted and fitted with Sigma Plot using the four-parameter equation for sigmoidal fit. The EC_{50} for PCP was 65 ± 4.25 nM ($P < 0.01$). (d) Time course for PCP-induced cleavage of procaspase-3. Both pro- and 17 kDa caspase-3 were probed on the same membrane. Data represent means ± SEM for 4–6 experiments (*$P < 0.05$; compared with control of each assay used, *t*-test).

PCP Decreases Phosphorylation of Akt^{Ser473}

Akt activity can be modulated by phosphorylation either on residue Thr308 or Ser473 of Akt (Brazil *et al*, 2004; Kumar and Madison 2005). It has been reported that phosphorylation of Thr308 is stimulated by DA receptor activation, while phosphorylation of Ser473 is regulated by NMDA receptor potentiation (Filippa *et al*, 1999; Brami-Cherrier *et al*, 2002; Papadia *et al*, 2005; Soriano *et al*, 2006). Thus, we hypothesized that PCP may decrease phosphorylation of Akt^{Ser473}. The effect of PCP (10 mg/kg) on pAkt^{Ser473} expression in rat pups (PN7) is shown in Figure 2a–c. Compared with rat pups injected with saline (10 ml/kg), pAkt^{Ser473} expression was significantly decreased in the frontal cortex (Figure 2a), hippocampus (Figure 2b), and striatum (Figure 2c). The reduction of pAkt^{Ser473} can be observed 3 h after PCP injection and it does not recover even after 9 h in any of the three brain areas. It is unknown whether this long-lived effect of PCP is due to the continued presence of PCP or to the slow recovery of this system following the initial insult. The metabolic half-life of PCP in adults is about 4.5 h (Shelnutt *et al*, 1999), but these data are unavailable for pups at this stage of maturity. This profile of pAkt^{Ser473} parallels the change in caspase-3 activation in rat pups treated with PCP observed previously by this laboratory (Wang and Johnson, 2007). The reduction in pAkt^{Ser473} was also observed in dissociated neuronal cultures (Figure 2d and e). In this preparation, the decrease of pAkt^{Ser473} can be measured as early as 1 h after PCP administration (data not shown), and remains low in the presence of PCP (Figure 2d). However, as shown in Figure 2, the total Akt protein does not change significantly either in rat pups or cultures. This suggests that Akt activity, as measured by pAkt^{Ser473}, is substantially downregulated by PCP.

Akt Inactivation Plays a Causal Role in PCP-Induced Neurotoxicity

It has been reported that activation of L-type calcium channels (LTCCs) can increase synaptic strength and favor cell survival through facilitating calcium-dependent signaling mechanisms that propagate signals to the nucleus and regulate gene transcription and expression (Marshall *et al*, 2003; Yano *et al*, 2005; Gomez-Ospina *et al*, 2006). We thus proposed that activating LTCCs should block PCP-induced neurotoxicity by increasing pAkt^{Ser473} expression. Cultured neurons were pretreated for 30 min with Bay K8644, an agonist of LTCCs, and then exposed to PCP for 48 h. This experiment showed that Bay K8644 blocked the effect of PCP on pAkt^{Ser473} reduction in a dose-dependent manner,

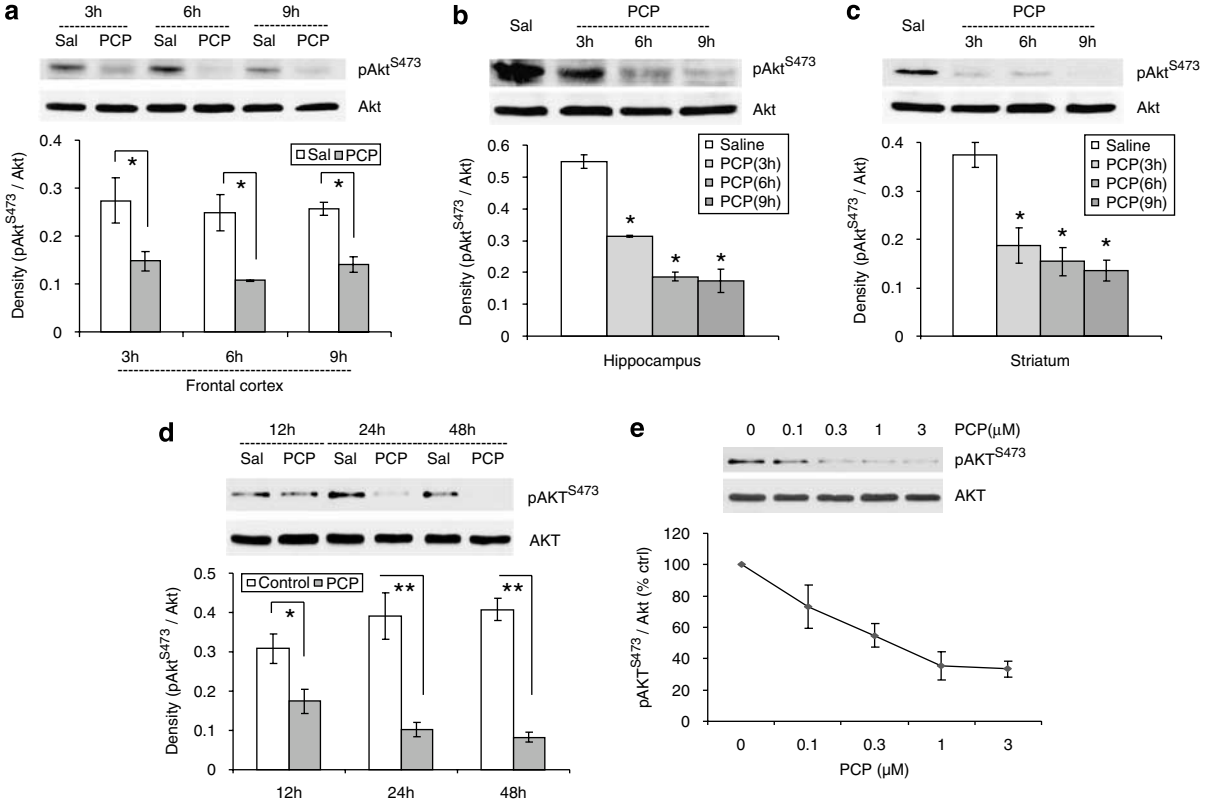

Figure 2 Temporal, regional, and dose-dependent inhibition of pAkt^{Ser473} (pAkt^{S473}) by PCP *in vivo* and *in vitro*. Rat pups (PN7) were administrated PCP 10 mg/kg and killed 3, 6, and 9 h following administration. Brain homogenate (40 μg) from each region was subjected to Western analysis. Blots were probed with antibodies against pAkt^{S473} and total Akt in the same membrane. Significant decreases of pAkt^{S473} relative to total Akt were observed 3, 6, and 9 h following PCP administration in the frontal cortex (a), hippocampus (b), and striatum (c). Controls shown for (b) and (c) were taken at 9 h, as there is no difference between controls at 3, 6, or 9 h. Similar decreases in pAkt^{S473} were observed in RIPA buffer lysates (30 μg) of neuronal cultures after exposure to PCP. (d) The time course of this decrease and (e) the concentration–response relationship as a percent of control after 48 h of treatment. Total Akt did not show obvious changes either *in vivo* or *in vitro*. Blots in upper of each panel in this figure are typical examples, whereas the lower portions of each graph are the results summarized from 3 to 5 experiments. These data represent means ± SEM (*$P<0.05$; **$P<0.01$ compared with saline treatment, ANOVA).

but did not affect total Akt (Figure 3a). Bay K8644 also prevents PCP-induced neurotoxicity in a concentration-dependent manner (Figure 3b). Bay K8644 (3 μM) had no effect on either measure alone (data not shown). Thus, the protection of Bay K8644 is consistent with the activation of $pAkt^{Ser473}$. To further confirm the role of Akt in Bay K8644 protection against PCP-induced toxicity, we inhibited Akt activity by two selective Akt inhibitors (VIII and X, 2 μM each). In the presence of either, the effect of Bay K8644 was completely eliminated (Figure 3c), suggesting that Akt activation is responsible for this protection. If PCP-induced neurotoxicity results from blockade of synaptic NMDA receptors, then potentiation of synaptic NMDA receptors should attenuate PCP-induced neurotoxicity. Thus, we used two approaches to activate synaptic NMDA receptors. First, 10 μM NMDA, a concentration that preferentially activates synaptic NMDA receptors (Soriano *et al*, 2006), was added to the cultures for 24 h. Then, after washout, the pretreated neurons were exposed to PCP and neurotoxicity was measured using both LDH and MTT assays (Figure 4a). Similarly, synaptic NMDA receptors were also activated by blocking GABAergic inhibition with co-application of bicuculline methobromide and 4-aminopyridine (4AP), a weak potassium channel blocker (Hardingham *et al*, 2002; Ivanov *et al*, 2006). These results (Figure 4a and b) showed that activation of synaptic NMDA receptors by either approach completely prevents PCP-induced neurotoxicity. In addition, $pAkt^{Ser473}$ expression was increased when synaptic NMDA receptors were activated by these approaches (insets in Figure 4a and b). As elevation of synaptic strength facilitates signaling cascades such as PI-3K-Akt and Ras-MEK-ERK1/2 pathways (Papadia *et al*, 2005; Soriano *et al*, 2006), we examined the effects of selective inhibitors of PI-3K, Akt, and MAPK to further confirm that the protection emanates from activation of synaptic NMDA receptors. The protection afforded by Bic + 4-AP was abolished completely in the presence of inhibitors of either PI-3K (wortmannin or LY294002) or Akt (VIII or X) (Figure 4c). However, the effect was only partially reversed by U0126, a MAPK inhibitor, suggesting that the protection afforded by synaptic NMDA receptor activation primarily involves activation of the PI-3K-Akt pathway, though the MAPK pathway may also play a role.

The Role of pGSK-3β^{Ser9} in PCP-Induced Neurotoxicity

Akt is one of principal kinase regulators of GSK-3β, inhibiting GSK-3β activity by phosphorylation of Ser9 on N terminus of GSK-3β (Cohen and Frame, 2001). Blockade of NMDA receptors has been shown to decrease $pAkt^{Ser473}$ (Figure 2), resulting in inactivation of Akt. Consequently, GSK-3β phosphorylation would be expected to be decreased

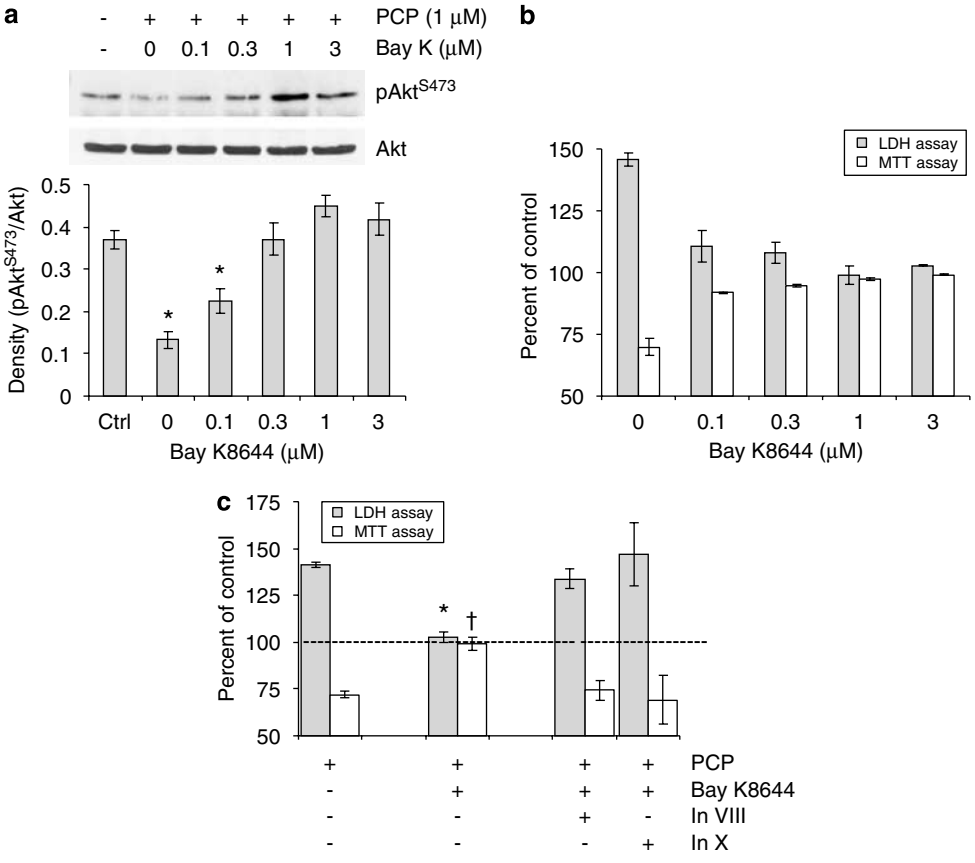

Figure 3 Activation of Akt by Bay K8644, an LTCC agonist, reverses PCP-induced neurotoxicity. Cultured neurons were pretreated with a series of Bay K8644 concentrations for 30 min and then exposed to PCP for 24 h for analysis of Akt phosphorylation (a), or 48 h in the presence or absence of various concentrations of Bay K8644 (b). (c) The reversal of the protective effects of 3 μM Bay K8644 by 60 min pretreatment with inhibitors of Akt, VIII (2 μM) or X (2 μM). Cell viability was measured by LDH release and MTT metabolism after 48 h. These data are from four experiments (*$P<0.05$, compared with pAkt/Akt control and LDH release in cells treated with PCP; †$P<0.05$, compared with MTT assay in PCP-treated cells, *t*-test).

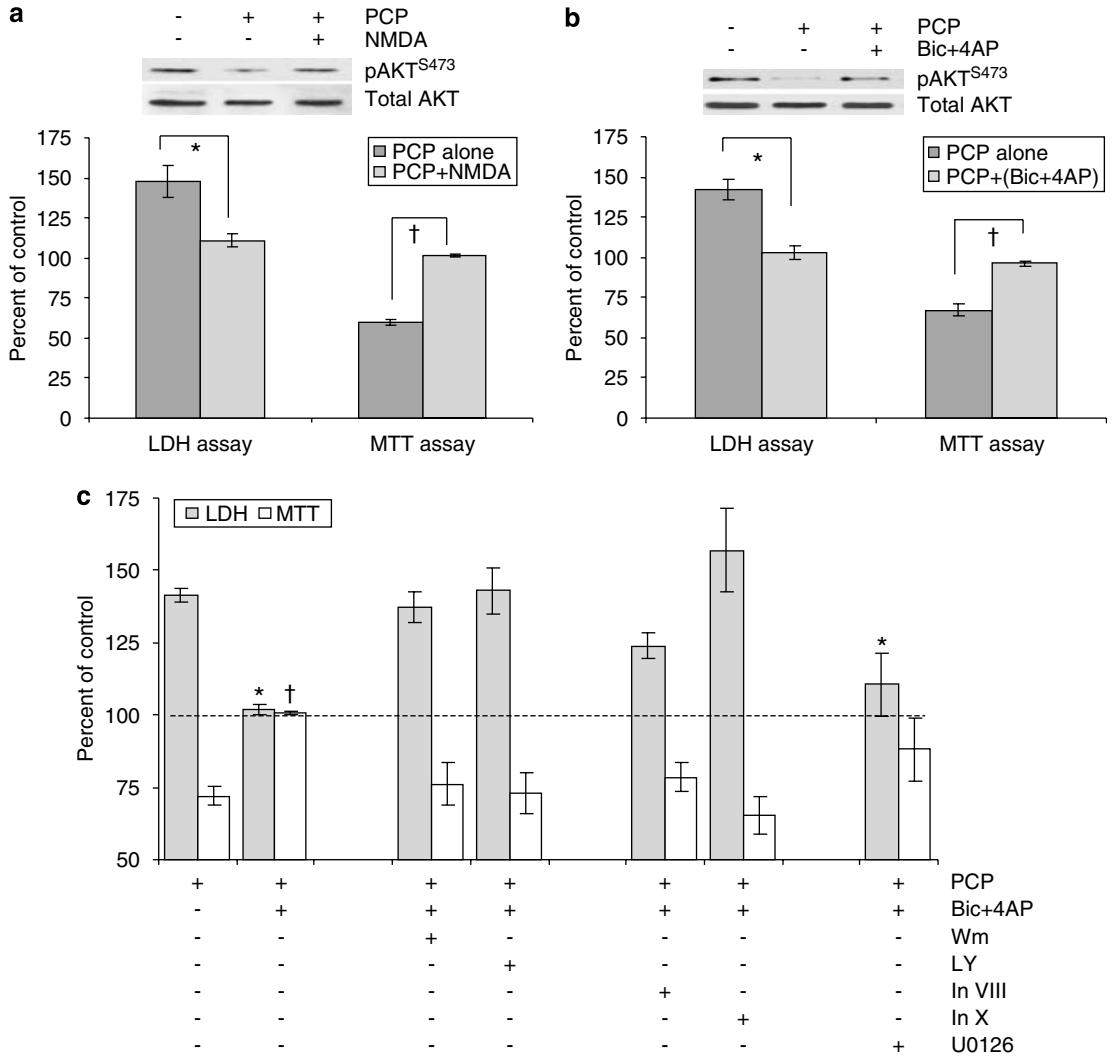

Figure 4 Activation of synaptic NMDA receptors prevents PCP-induced neurotoxicity. Cultured neurons were treated with either bath application of NMDA (10 μM) for 24 h, followed by washout and replacing with fresh medium (a) or the addition of bicuculline (50 μM) plus 4-aminopyridine (25 μM) (Bic + 4-AP) overnight (b). Treated cells were exposed to PCP for 48 h, followed by examining the cell viability. Insets in (a) and (b) show the effects of these treatments on $pAkt^{Ser473}$ ($pAkt^{S473}$) protein by WB. (c) The role of PI-3K, Akt, and MAPK in the neuroprotection afforded by treatment of cultured neurons with Bic + 4-AP for 24 h to activate synaptic NMDA receptors was examined by treatment with the PI-3K inhibitors, wortmannin (0.15 μM) or LY294002 (150 μM), the Akt inhibitor, IIIV (2 μM) or X (2 μM), and the MAPK inhibitor, U0126 (5 μM) for 60 min before PCP treatment for 48 h. Cell viability was determined and summarized from four independent experiments. Data represent mean ± SEM (*$P<0.05$, compared with LDH in cells treated with PCP alone; †$P<0.05$, compared with MTT in PCP-treated cells, *t*-test).

and its activity increased as a result. GSK-3β has been shown to act as an apoptosis-inducing kinase in the nervous system (Hetman *et al*, 2002; Szatmari *et al*, 2005; Rivero Vaccari *et al*, 2006). Accordingly, we proposed that PCP would decrease the expression of $pGSK\text{-}3\beta^{Ser9}$ (increasing its activity), an effect that could account for PCP-induced neurotoxicity. First, rat pups (PN7) were treated with PCP and tissues were collected from rat brain as described for Akt measurements. In parallel with alteration of $pAkt^{Ser473}$ by PCP, $pGSK\text{-}3\beta^{Ser9}$ was shown to be substantially decreased following PCP either *in vivo* or *in vitro* (Figure 5). Both the frontal cortex (Figure 5a) and the hippocampus (Figure 5b) showed marked PCP-induced reductions in $pGSK\text{-}3\beta^{Ser9}$, but in striatum the decrease of $pGSK\text{-}3\beta^{Ser9}$ was more evident (Figure 5c), with the ratio of $pGSK\text{-}3\beta^{Ser9}$/total GSK-3β being 0.161 and 0.151 for 6 and 9 h exposure, while these ratios were only 0.55 and 0.40 for the hippocampus and 0.33 and 0.25 for frontal cortex, respectively, over the same time frame. The reason for these regional differences is unknown. Interestingly, it agrees with a previous report from this laboratory that the striatum was more vulnerable to acute PCP exposure (Wang and Johnson, 2005). Second, cultured neurons were exposed to PCP and it was also found to robustly reduce $pGSK\text{-}3\beta^{Ser9}$ (Figure 5d). It has been reported that caspase-3 activation plays a major role in PCP-induced neurotoxicity (Wang and Johnson, 2007). Caspase-3 is downstream of, and activated by, GSK-3β (Song *et al*, 2002). Therefore, we examined in cultured neurons the relationship between GSK-3β and caspase-3 activation. These results demonstrated that the $pGSK\text{-}3\beta^{Ser9}$ reduction can be observed 1 h after PCP treatment with the peak between 12 and 24 h, which correlates well to temporal profiles of caspase-3 activation (Figure 5e).

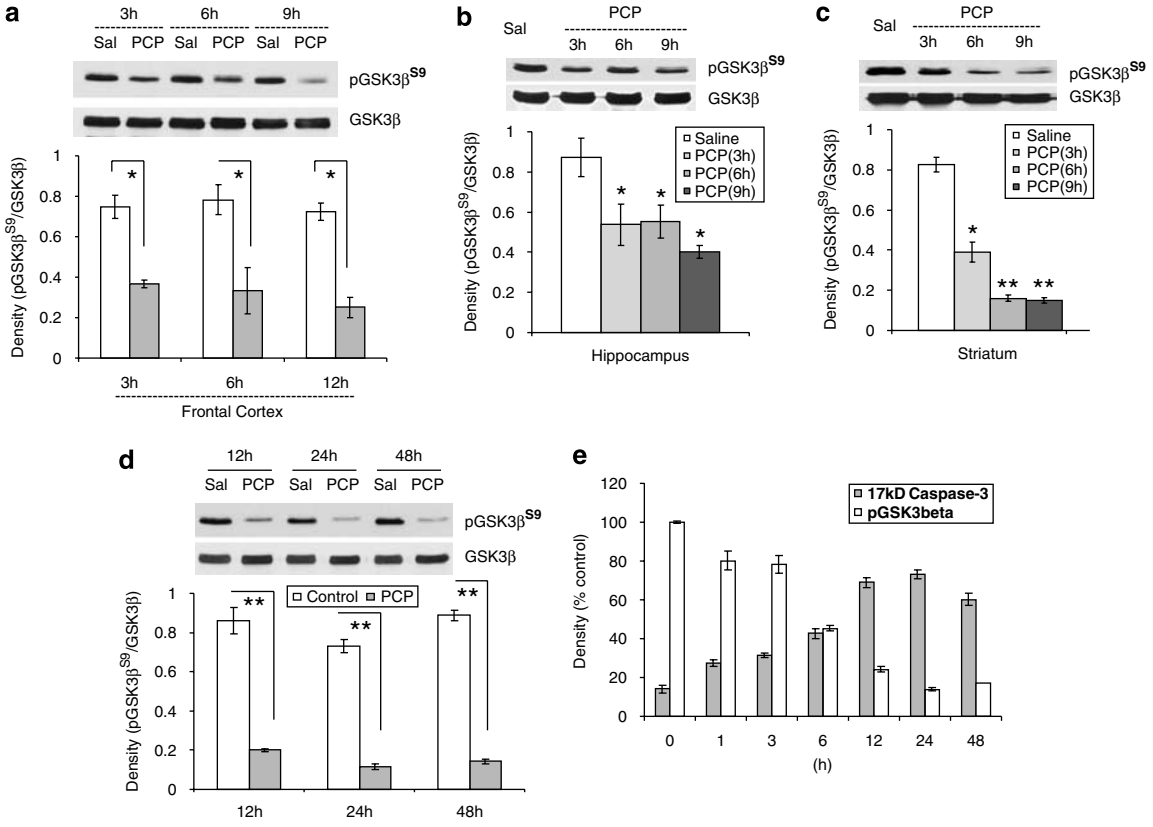

Figure 5 PCP decreases pGSK-3β^{Ser9} (pGSK-3β^{S9}) *in vivo* and *in vitro*. Rat pus and cultured cells were treated as described for the Akt experiment in the legend of Figure 2. A decrease in pGSK-3β^{S9} was found in the frontal cortex (a), hippocampus (b), and striatum (c), as well as in cultured neurons (d) compared with saline treatment. Control values for (b) and (c) are taken at 9 h. Examples of WBs are shown in the upper portion of panels a, b, c, and d. Summary data are shown from 3 to 5 experiments in the lower portion of each corresponding panel. No significant change of total GSK-3β was found. (e) The relative density of activated (17 kDa) caspase-3 and pGSK-3β^{S9} in WBs from cell lysates (30 μg) from cultured neurons treated with 1 μM PCP for different times (0–48 h). These data were summarized from three independent experiments (*$P<0.05$; **$P<0.01$; compared with saline control, ANOVA).

To further clarify the role of GSK-3β in PCP-induced neurotoxicity, we tested two relatively selective GSK-3β inhibitors, lithium chloride and kenpaullone. PCP-induced neurotoxicity was completely reversed by either lithium chloride or kenpaullone (Figure 6a). In terms of inhibitor selectivity, lithium chloride has been reported to inhibit several other protein kinases, such as PKA, Akt, PKC, etc with only slightly less potency than that for GSK-3β (Davies *et al*, 2000). Similarly, kenpaullone, which has an IC_{50} of 0.23 μM for GSK-3β and 0.67 μM for CDK2/cyclin A (Bain *et al*, 2003). To exclude the potential non-selective effects of these inhibitors, we used a 48-h treatment with siRNA directed against GSK-3 to verify the role of GSK-3β in PCP-induced neurotoxicity. GSK-3β expression was shown to be significantly inhibited by 100 nM GSK-3 siRNA and was nearly completely suppressed by 150 nM (Figure 6b). Caspase-3 activation and consequent neuronal apoptosis induced by PCP were essentially prevented by GSK-3 siRNA (150 nM) (Figure 6c and d).

DISCUSSION

These data significantly expand on recent work demonstrating that ketamine-induced apoptosis in culture was attenuated by GSK-3 inhibitors (Takadera *et al*, 2006). The current study demonstrates that PCP-induced cell death results from blockade of synaptic NMDA receptors and that this can be prevented by enhancing synaptic strength. Inhibition of the PI3K-Akt-GSK-3β signaling pathway plays major role in neurodegeneration caused by PCP or other NMDA channel blockers. Activation of Akt by stimulating synaptic NMDA receptors or by activating calcium influx through LTCCs favors cell survival. It was also shown that PCP-induced neurotoxicity can be prevented by blocking GSK-3β dephosphorylation by lithium and other selective GSK-3β antagonists, including siRNA. Finally, the present data provide a mechanism by which NMDA receptor hypofunction could lead to neuronal death during development. If this was of sufficient magnitude, it could lead to behavioral abnormalities later in life and could reasonably contribute to the behavioral pathology associated with schizophrenia.

It has been reported that caspase-3 can be activated by low $[Ca^{2+}]_i$, and that NMDAR antagonists lead to reduction of $[Ca^{2+}]_i$, activation of caspase-3, and cell death (Han *et al*, 2001; Takadera *et al*, 1999; Turner *et al*, 2002; Yoon *et al*, 2003). However, whether PCP-induced reduction of $[Ca^{2+}]_i$ is actually the cause of caspase-3 activation and subsequent cell death is uncertain and there is some debate about the reduction of $[Ca^{2+}]_i$. For example, Canzoniero *et al* (2004) showed that cultures treated with MK801 and CNQX for

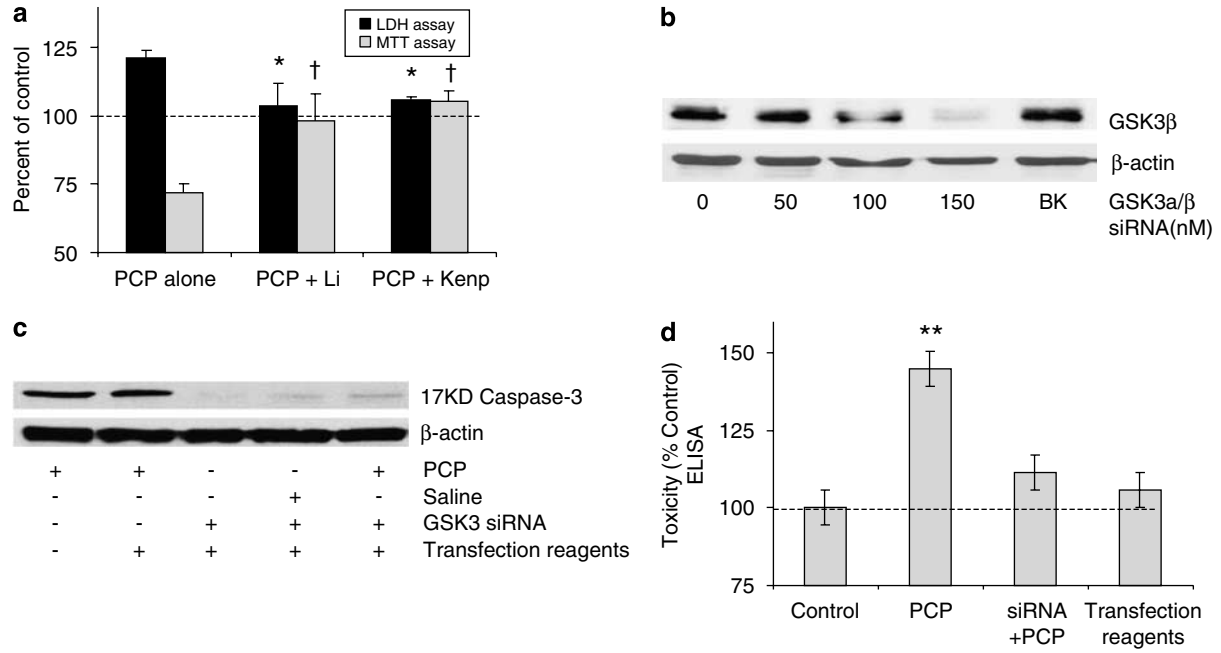

Figure 6 GSK-3β inhibition prevents PCP-induced neurotoxicity and caspase-3 activation. (a) Cultured neurons were pretreated for 1 h with the selective GSK-3β inhibitors, lithium chloride (2 mM), or kenpaullone (5 μM), and then exposed to PCP for 48 h. The summary data shown in (a) are from four experiments. (b) This WB shows that GSK-3 siRNA diminished GSK-3β expression in a concentration-dependent manner. Cultured neurons were transfected with the indicated concentration of GSK-3a/β siRNA, lysed after 48 h incubation, and subjected to WB using an antibody against total GSK-3β to examine the effect of silencing of GSK-3β expression. β-Actin was used for a loading control and siRNA specificity. Cells (DIV12) were transfected with 150 nM of GSK-3β siRNA and incubated in culture medium for 48 h, followed by exposure to PCP for 24 h. Activated caspase-3 was determined by WB using cell lysate (30 μg protein) from culture neurons transfected with siRNA (c), and neuronal viability was determined by a Cell Death ELISA kit using the same neuronal lysate (*$P<0.05$, compared with LDH release in PCP group; $^{\dagger}P<0.05$, compared with MTT in PCP group; **$P<0.01$, compared with control in Cell Death ELISA, *t*-test).

110 min did not exhibit any decrease in $[Ca^{2+}]_i$. The reason for this apparent conflict is unknown, but the route of Ca^{2+} entry and subsequent subcellular compartmentalization may play a critical role in determining which signaling pathways are activated. Calcium entry through LTCCs activates Akt (Murphy *et al*, 1991) and various transcription factors and kinases such as CREB, BDNF, IGF-1, PI-3K, Akt, and Src (Blair and Marshall, 1997; Graef *et al*, 1999; Dolmetsch *et al*, 2001; Ikegami and Koike, 2000) which, in turn, help to determine cell fate (Morgan and Curran, 1986; Mao *et al*, 1999). This may explain why inhibition of Akt can block the protection afforded by LTCCs. Furthermore, Ca^{2+} entry through synaptic NMDA receptors may induce CREB activity and *BDNF* gene expression, while Ca^{2+} entry through extrasynaptic NMDA receptors is known to activate a general and dominant CREB shut-off pathway that blocks the induction of *BDNF* expression (Hardingham *et al*, 2002). Thus, blockade of synaptic calcium entry may well be injurious, but blockade of extrasynaptic Ca^{2+} entry could even be protective by preventing NMDA receptor-mediated loss of mitochondrial membrane potential and cell death (Hardingham *et al*, 2002; Vanhoutte and Bading, 2003). Therefore, a simple reduction in intracellular Ca^{2+} is insufficient to fully explain the mechanism of cell death resulting from NMDAR blockade.

Enhancement of synaptic strength activates cell survival signaling cascades, such as the PI-3K-Akt and Ras-MEK-ERK1/2 pathways, promoting long-term neuroprotection against a variety of insults, including trophic deprivation and protein kinase inhibition (Soriano *et al*, 2006; Ivanov *et al*, 2006). Blockade of NMDA receptors can impair synaptic plasticity, eliminating signaling through the NMDA receptor. Hansen *et al* (2004) demonstrated that administration of MK801 to rat pups (PN7) led to reduced levels of BDNF and subsequent signaling through Ras-MEK-ERK in brain regions displaying apoptosis. Although exogenous BDNF prevented MK801-induced toxicity in that study, transgenic *Ras* activation only prevented about 40% of MK801-induced neurotoxicity (Hansen *et al*, 2004), strongly suggesting the existence of other pathways, such as the activation of Akt-GSK-3β signaling as implicated in the current study. Although not investigated here, activation of this pathway could provide protection through its phosphorylation and inhibition of proapoptotic mediators such as Bad, FOXO family members, IκB kinase-β (Datta *et al*, 1999). Moreover, Akt phosphorylates GSK-3β with the consequence of reducing GSK-3β activity and favoring cell survival.

Activation of GSK-3β has been reported to promote apoptosis in a wide variety of conditions (Bijur and Jope, 2001; Jope and Johnson, 2004). Previous reports have demonstrated that blockade of NMDA receptors results in an increase in proapoptotic Bax and a decrease in antiapoptotic Bcl-X_L and Bcl_2 (Wang *et al*, 2000; Hansen *et al*, 2004). Moreover, it has been shown that GSK-3β can activate Bax by direct phosphorylation of Bax-α on serine 163, a residue found within a putative GSK-3β phosphorylation motif (Linseman *et al*, 2004). This suggests that

GSK-3β may exert its proapoptotic effects in neurons by modifying Bcl_2 family member expression, which, in turn, can lead to impairment of mitochondrial membrane potential, cytochrome *c* release, and caspase-3 activation.

Akt phosphorylation could also play a role in integrating input from both dopaminergic and glutamatergic influences and, as such, could be a key player in the pathogenesis of schizophrenia. Two key phosphorylation sites, Thr308 and Ser473, are critical in regulating Akt activity (Brazil *et al*, 2004; Kumar and Madison, 2005). These modifications have been reported to involve different signaling pathways. Activation of Akt by phosphorylation of Thr308 is PI-3K independent, and regulated by PKA (cAMP-dependent protein kinase) (Filippa *et al*, 1999), while Brami-Cherrier *et al* (2002) showed that DA D1 and D2 agonists increase phosphorylation levels of Akt on Thr308 (but not Ser473), independently of PI-3K signaling. Furthermore, Beaulieu *et al* (2004) proposed that increased DA neurotransmission in mouse striatum activates an alternative pathway involving phosphorylation of pAktThr308 and subsequent phosphorylation of GSK-3α/βS21/9. Interestingly, activation of NMDA receptors also increases Akt activity, but by phosphorylation of Ser473 rather than Thr308. Opposite to DA activation, NMDA receptors activation occurs in a PI-3K-dependent manner (Sutton and Chandler, 2002; Papadia *et al*, 2005; Soriano *et al*, 2006). The current study demonstrates that blockade of NMDA receptors by PCP decreases Akt activity by reducing $pAkt^{Ser473}$ and increases GSK-3β activity by reducing $pGSK\text{-}3\beta^{Ser9}$. Importantly, this effect of PCP, as well as PCP's neurotoxicity, can be reversed by increasing the activity of synaptic NMDA receptors, thereby supporting the hypothesis that PCP's effects are mediated by blockade of synaptic, rather than extrasynaptic, NMDA receptors. This dual regulation of phosphorylation of Akt and $GSK\text{-}3\beta^{Ser9}$ could be important in both the etiology and therapy of schizophrenia (Kalkman, 2006; Kozlovsky *et al*, 2006).

It should be mentioned that these data appear to contradict two previous studies showing that administration of PCP or MK801 to adult mice or rats increased $pAkt^{Ser473}$ and/or $pGSK\text{-}3\beta^{Ser9}$ (Svenningsson *et al*, 2003; Ahn *et al*, 2005). This difference could be due to the difference between adults and developing rat pups, but it also could be explained by a biphasic dose response to MK801. That is, Ahn *et al* (2005) reported that doses up to 1 mg/kg MK801 increased $pAkt^{Ser473}$, but higher doses resulted in a decrease in $pAkt^{Ser473}$. Thus, it is likely that the dose of PCP used in this study corresponds to the higher doses of the more potent MK801 used by Ahn *et al* (2005).

Further study of Akt-GSK-3β signaling and its role in various animal models of schizophrenia appear to be a promising approach to the discovery of novel therapeutics for schizophrenia and possibly related illnesses such a mania. Altered Akt-GSK-3β signaling cascade has been implicated in the pathogenesis of schizophrenia (Norton *et al*, 2006; Ross *et al*, 2006) and it has been demonstrated that GSK-3β is substantially lower in post-mortem frontal cortex of schizophrenics (Kozlovsky *et al*, 2002, 2005). Also, Emamian *et al* (2004) reported that $pGSK\text{-}3\beta^{Ser9}$ and Akt1 were significantly decreased in the peripheral lymphocytes and brains of schizophrenics. This group also reported an association between schizophrenia and an Akt1 haplotype associated with lower Akt protein levels and a greater sensitivity to the sensorimotor gating-disruptive effect of amphetamine. This supports the notion that defective Akt1-GSK-3β signaling could contribute to the pathogenesis of schizophrenia. In fact, an association of the Akt1 gene with schizophrenia has been reported in Japanese (Ikeda *et al*, 2004), European (Schwab *et al*, 2005), and Iranian populations (Bajestan *et al*, 2006), although other studies have not replicated these observations (Ohtsuki *et al*, 2004; Ide *et al*, 2006). In addition, Lai *et al* (2006) reported that *Akt1*-deficient mice displayed altered prefrontal neuronal architecture and also abnormal performance in a working memory task, generally regarded as a core feature of schizophrenia (Lewis and Gonzalez-Burgos, 2006). Thus, the current study supports the potential role of altered Akt1-GSK-3β signaling in schizophrenia and suggests that further study of the molecular mechanisms of NMDA receptor regulation of this pathway could provide additional useful insights into the pathology and treatment of this disease and potentially other neurodegenerative diseases as well.

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FINANCIAL DISCLOSURES/CONFLICT OF INTEREST

Dr Lei reports no biomedical financial interests or potential conflicts of interest. Ms Xia reports no biomedical financial interests or potential conflicts of interest. Dr Johnson reports no biomedical financial interests or potential conflicts of interest.

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