



Published in final edited form as:

Neuropharmacology. 2009 January ; 56(1): 47–55. doi:10.1016/j.neuropharm.2008.08.015.

Glutamate Receptors as Targets of Protein Kinase C in the Pathophysiology and Treatment of Animal Models of Mania

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Summary

Considerable biochemical evidence suggests that the protein kinase C (PKC) signaling cascade may be a convergent point for the actions of anti-manic agents, and that excessive PKC activation can disrupt prefrontal cortical regulation of thinking and behavior. To date, however, brain protein targets of PKC's anti-manic effects have not been fully identified. Here we showed that PKC activity was enhanced in the prefrontal cortex of animals treated with the psychostimulant amphetamine.

Phosphorylation of MARCKS, a marker of PKC activity, was increased in the prefrontal cortex of animals treated with the psychostimulant amphetamine, as well as in sleep-deprived animals (another animal model of mania), but decreased in lithium-treated animals. The antidepressant imipramine, which shows pro-manic properties in patients with bipolar disorder (BPD), also enhanced phospho-MARCKS in prefrontal cortex in vivo. We further explored the functional targets of PKC in mania-associated behaviors. Neurogranin is a brain-specific, postsynaptically located PKC substrate. PKC phosphorylation of neurogranin was robustly increased by pro-manic manipulations and decreased by anti-manic agents. PKC phosphorylation of the NMDA receptor site NR1S896 and the AMPA receptor site GluA1T840 was also enhanced in the prefrontal cortex of animals treated with the antidepressant imipramine, as well as behaviorally sleep-deprived animals, in striking contrast to the reduced activity seen in lithium-treated animals. These results suggest that PKC may play an important role in regulating NMDA and AMPA receptor functions. The biochemical profile of the PKC pathway thus encompasses both pro- and anti-manic effects on behavior. These results suggest that PKC modulators or their intracellular targets may ultimately represent novel avenues for the development of new therapeutics for mood disorders.

Keywords

Protein kinase C (PKC); mania; lithium; neurogranin; NR1; GluA1

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Conflict of Interest We would like to acknowledge the support of the Intramural Research Program of the National Institute of Mental Health. The author(s) declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Introduction

Bipolar disorder (BPD) is a chronic, severe, often life-threatening illness (Goodwin and Jamison, 2007) that affects approximately 1-2% of the US population (Judd and Akiskal, 2003). Although genetic factors play a major role in the etiology of BPD, the biochemical abnormalities underlying the predisposition to, and the pathophysiology of, this disorder remain to be fully elucidated. Recently, accumulating data have implicated glutamatergically-mediated synaptic plasticity in the treatment of BPD (Coyle and Duman, 2003; Du et al., 2008; Du et al., 2003; Du et al., 2004a; Kugaya and Sanacora, 2005; Maeng et al., 2008; Nestler and Carlezon, 2006; Sanacora et al., 2008; Young, 2007). Given that alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptor trafficking is critical for regulating various forms of synaptic plasticity in the CNS, its potential involvement in the pathophysiology or treatment of certain neuropsychiatric disorders is an emerging area of interest (Barbon et al., 2006; Brebner et al., 2005; Carlezon and Nestler, 2002; Chao et al., 2002; Peineau et al., 2007; Pittaluga et al., 2007; Self, 2002; Snyder et al., 2000).

In recent years, research has increasingly investigated the role of intracellular signaling cascades in the pathophysiology and treatment of BPD (Manji et al., 2003; Zarate et al., 2006). One signaling cascade that has been studied with considerable interest is the protein kinase C (PKC) signaling pathway. It has a heterogeneous distribution in brain, and plays a major role in the regulation of neuronal excitability, neurotransmitter release, and long-term alterations in gene expression and plasticity (Huang, 1990; MacDonald et al., 2001; Nogues, 1997; Ramakers et al., 1997). A growing body of data has also implicated dysregulation of the PKC signaling cascade in the pathophysiology of BPD (Hahn and Friedman, 1999; Jope et al., 1996; Mathews et al., 1997; Turecki et al., 1998; Wang and Friedman, 1996). Specifically, PKC overactivity has been associated with motoric hyperactivity, risk-taking behavior, and excessive hedonic drive, all of which are cardinal symptoms of mania. Notably, the current mainstays in the treatment of mania—the structurally dissimilar anti-manic agents lithium (a monovalent cation) and valproate (VPA, a small fatty acid)—indirectly inhibit PKC (Chen et al., 2000a). In addition, clinical studies demonstrate that the PKC inhibitor tamoxifen has anti-manic efficacy in human manic subjects (Yildiz et al., 2008; Zarate et al., 2007). Overactive PKC signaling in the prefrontal cortex may thus explain many of the symptoms of mania. Notably, imaging studies have demonstrated marked under-activity of the right prefrontal cortex in mania (Arnsten and Manji, 2008); furthermore, severe dysfunction of the prefrontal cortex, particularly in the right hemisphere, is highly consistent with the symptoms of mania (Arnsten and Manji, 2008).

To date, however, the precise cellular targets of PKC that mediate these complex behavioral effects have not been fully elucidated. We therefore undertook a series of biochemical studies to investigate the potential role of PKC, and its targets, in the development and treatment of mania. Toward this end, we assessed the role of PKC phosphorylation of 1) NMDA and AMPA receptors; 2) neurogranin, which is a postsynaptically located and brain-specific PKC substrate; and 3) myristoylated alanine-rich C kinase substrate (MARCKS), an intracellular structural PKC substrate implicated in BPD, in a sleep deprivation model of mania, as well as in animals undergoing sustained treatment with the pro-manic agent amphetamine, the antidepressant imipramine, and the anti-manic agent lithium.

Methods

Treatment of animals with amphetamine, imipramine and lithium

All animal treatments, procedures, and care were approved by the National Institute of Mental Health (NIMH) Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals* (ISBN 0-309-05377-3). The mice were acclimated to the laboratory for a week before the experiment. Male C57/B6 mice (seven to eight weeks; Harlan, Indianapolis, IN) were housed three to four per cage in a 12 hour light/dark cycle and had access to water and food *ad libitum*. Amphetamine or imipramine-treated animals were given injections of imipramine (10 mg/kg in 0.3 ml of saline), amphetamine (2.5mg/kg in 0.3 ml of saline), or saline (twice daily, i.p.) for 10 days. For lithium treatment, C57BL/6 mice were fed with chow containing a low dose of lithium (Bio-Serv, Frenchtown, NJ, Li₂CO₃-1.2g /kg) for one week, followed by full dose lithium chow (Bio-Serv, Frenchtown NJ, Li₂CO₃-2.4g/kg) for 3 weeks. Control animals received control chow. Animals treated with lithium-containing chow were also provided with a bottle of saline in addition to drinking water. Blood samples were taken at the time of decapitation and analyzed for lithium concentration (0.89±0.25mM). After drug treatment, the prefrontal cortices from mouse brains were removed immediately after decapitation, immersed in liquid nitrogen, placed in labeled Eppendorf tubes on dry ice, and then stored and kept frozen at -80°C until processing.

Sleep deprivation

Male Wistar-Kyoto rats (Harlan, 11-12 week old at time of surgery) were used. Under deep isoflurane anesthesia (1.5-2% volume) to avoid damage to the left cortical hemisphere (used for the molecular studies), epidural screw electrodes were implanted over the right parietal and occipital cortex, and cerebellum (reference electrode). Electrodes were fixed to the skull with dental cement. Two stainless steel wires (diameter 0.4 mm) were inserted into the neck muscles to record the electromyogram (EMG).

After surgery, all rats were housed individually in transparent Plexiglas cages (36.5 × 25 × 46 cm), and kept in sound-proof recording boxes for the duration of the experiment. Lighting and temperature were kept constant (12 hour light/dark cycle, lights on at 10 am, 23 ± 1°C; food and water were available *ad libitum* and replaced daily at 10 am). Seven to ten days were allowed for recovery after surgery, and experiments were started only after the sleep/wake cycle had fully normalized. The rats were connected by means of a flexible cable to a commutator (Airflyte, Bayonne, NJ) and recorded continuously for two to four weeks using a Grass electroencephalograph (mod. 15LT, Astro-Med Inc., West Warwick, RI). Video recordings were performed continuously with infrared cameras (OptiView Technologies, Inc, Potomac Falls, VA) and stored in real time (AVerMedia Technologies, Inc, Milpitas, CA).

EEG and EMG signals were amplified (amplification factor 2000), and filtered [attenuation 50 % amplitude (- 6 dB)] as follows: EEG: high-pass filter at 0.1 Hz, low-pass filter at 30 Hz; EMG: high-pass filter at 10 Hz, low-pass filter at 100 Hz. All signals were sampled and stored at 128 Hz resolution. Sleep stages were scored off-line by visual inspection of 4-s epochs (SleepSign, Kissei Comtec, Nagano, Japan). Wakefulness was characterized by a low-voltage, high-frequency EEG pattern and phasic EMG-activity. Non-rapid eye movement (NREM) sleep was characterized by the occurrence of high-amplitude s low waves, spindles, and low tonic EMG activity. In contrast, during REM sleep, the EEG was similar to that during waking, but only heartbeats and occasional twitches were evident in the EMG signal. Vigilance state could always be determined. All animal procedures followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Facilities were reviewed and approved by the IACUC of the University of Wisconsin-Madison, and were inspected and accredited by the

Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Each day from 10 to 10:30 am all rats were gently handled and exposed to a new object to become familiar with the sleep deprivation procedure (these objects were not reused). Two groups of rats were used. Sleeping rats (S) were sacrificed during the light hours (around 2.30 pm) at the end of a long period of sleep (at least 45 minutes, interrupted by periods of waking no longer than two minutes) after spending at least 75% of the previous four hours asleep. Sleep deprived (SD) rats were sacrificed during the light period at the same time of day as S rats (around 2.30pm) after four hours of continuous wakefulness by exposure to novel objects. Novel objects included nesting and bedding material from other rat cages, wooden blocks, small rubber balls, plastic, metallic, wooden, or paper boxes and tubes of different shapes and colors. The boxes often had holes through which the rat could reach for a palatable food pellet or a paper towel. During the sleep deprivation experiment, a new object was introduced into the cage whenever the rat appeared drowsy and slow waves became apparent on the EEG. The rats were never disturbed when they were spontaneously awake, feeding, or drinking.

Rats were deeply anesthetized with isoflurane (within one minute) and decapitated. The head was cooled in liquid nitrogen and the whole brain was removed. Left cerebral cortex was dissected and divided into three regions (frontal, parietal, occipital). Samples were immediately frozen on dry ice and stored at -80°C .

After a one-week accommodation period, the lithium-treated mice received lithium-containing chow (custom-produced by Bio-Serve, Frenchtown, NJ). Drug-containing chow and control chow were identical, with the exception of the added drug, and were produced at both a low and regular concentration (1.2 and 2.4 g/kg, respectively). These doses of lithium have been used extensively by our group and others and have been found to lead to serum drug levels similar to those achieved therapeutically in the treatment of BPD (Yuan et al., 1999). Mice were initially treated for one week at the lower dose (to acclimatize them to the diet and reduce risks of side effects), followed by three weeks of the higher-dose treatment. The lithium experiment included 12 control and 12 experimental animals, all provided with an extra bottle of saline and daily bedding changes to minimize the effects of lithium-induced polyuria (a well-known side effect of lithium).

All mice were weighed and then killed by decapitation during the morning hours. Trunk blood was collected, and drug serum levels were performed by Medtox Laboratories (St. Paul, MN). Only animals with drug levels within the therapeutic range were used for additional studies. In general, ~80% of animals achieved therapeutic levels of lithium. Serum lithium blood levels of the animals used for additional analyses were 0.80 ± 0.13 mEq/L. Hippocampal tissue was dissected immediately after decapitation. Brain specimens were frozen rapidly in liquid nitrogen and stored at -80°C until further analysis.

Preparation of crude homogenates, cytosolic, and membrane fractions

The frontal cortices and hippocampi from adult mouse brains (three to four months old) were removed immediately after decapitation, immersed in liquid nitrogen, placed in labelled Eppendorf (Westbury, NY) tubes on dry ice and then stored and kept frozen at -80°C until processing. Samples were sonicated in Homogenization Buffer A (50 mM Tris-Cl, pH 7.5, containing 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 50 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 50 mM KF, 50 nM okadaic acid, 1mM sodium orthovanadate, 5mM sodium pyrophosphate, 0.1% NP-40, and 5 $\mu\text{g}/\text{ml}$ each of leupeptin, aprotinin, chymostatin, and pepstatin A), spun in the Eppendorf 5810R centrifuge (Westbury, NY) for 20 minutes at 4°C at 20,000 rcf, and the clear homogenate was used as total protein.

For the PKC assay and determination of phosphorylated and total myristoylated alanine-rich C kinase substrate (MARCKS) levels in the frontal cortex (see below), samples were separated into membranous and cytosolic fractions, because translocation of this structural and signalling protein into different cellular compartments offers insight into activity states. These samples were sonicated in Homogenization Buffer B (50 mM Tris-Cl, pH 7.5, containing 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 50 μ M 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride, 50 mM KF, 50 nM okadaic acid, 1mM sodium orthovanidate, 5mM sodium pyrophosphate, and 5 μ g/ml of leupeptin) centrifuged at $100,000 \times g$ for 30 minutes at 4°C in an Optima™ Max Ultracentrifuge (Beckman Coulter, Fullerton, CA) to yield the cytosol fraction, and aliquots of the supernatant were stored at -70°C until experimentation. The pellet fractions were re-suspended in Homogenizing Buffer B containing 0.1% Nonidet P-40, sonicated, and centrifuged again. Aliquots of the resulting supernatants were taken as the membrane fractions and stored at -80°C until further analysis.

PKC activity assay

PKC activity was determined in both the cytosolic and membrane fractions as previously described (Wu et al., 2002). Briefly, measurement was at 30°C for five minutes in a mixture (25 μ l) containing 30 mM Tris-Cl (pH 7.5), 6 mM MgCl_2 , 120 μ M [-32P] ATP, 1.0 mg/ml bovine serum albumin, 20 μ M Ng-(28-43) peptide substrate, 0.1 mg/ml phosphatidylserine, 0.02 mg/ml 1,2-dioleoylglycerol, 0.4 mM CaCl_2 or 1.5 mM EGTA, and 1 μ g protein of tissue extract. Reactions were stopped by tubes being placed at 99°C for five minutes, then allowed to cool for 10 minutes. Tubes were centrifuged with an Eppendorf centrifuge 5415D (Westbury, NY) for three minutes and 5 μ l of reaction mixture was blotted on 4×4 cm Whatman P81 chromatography paper. Blotted paper was allowed to dry for 30 minutes and then washed in a 7% phosphoric acid solution three times for 10 minutes each. The paper was placed on aluminum foil and allowed to dry overnight. ^{32}P -labeled peptide substrates blotted on chromatography paper were measured in a scintillation counter. All reactions were run in duplicate or triplicate. Activities were converted to picomoles of ^{32}P -incorporated into peptide/ μ g of protein/min. These values were then expressed as percent of controls to allow comparison.

Western blotting analysis

Aliquots of crude whole-cell homogenates, membrane fractions, or cytosol fractions taken from the frontal cortices of mice were used to determine phosphorylated and total protein. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay kit, and the linearity of the protein concentration for immunoblotting was ascertained by resolution of selected concentrations of protein. Equal amounts of proteins were subjected to 10% SDS-PAGE gels and separated by electrophoresis. Proteins were then electrophoretically transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose was blocked with Tris Buffered Saline plus Tween 20 (TBST), 10% nonfat dry milk, and then incubated with anti-Neurogranin antibody (Upstate Laboratories, Syracuse, NY), anti-phospho-neurogranin (Ser 36) (Upstate Laboratories, Syracuse, NY), anti-actin antibody (Chemicon, Temecula, CA) anti-ATPase (Na+K) (Abcam, Cambridge, UK), anti-MARCKS antibody (Calbiochem, Gibbstown, NJ); anti-Phospho-MARCKS (Ser 152/156) antibody (Cell Signaling Technology, Danvers, MA), anti-phospho-NR1S986 (Upstate Laboratories, Syracuse, NY), anti-NR1 (Chemicon, Temecula, CA), anti-phospho-GluA1T840 (Abcam, Cambridge, MA), anti-phospho-GluA831 (Affinity BioReagents, Golden, CO), and anti-GluA1 (Chemicon, Temecula, CA). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Cell Signaling Technology, Danvers, MA). The ECL plus kit (GE Healthcare, Piscataway, NJ) was used as a detection system. Similarly, membrane and cytosol fractions from the frontal cortices of mice were used to determine levels of phosphorylated and non-phosphorylated MARCKS with specific antibodies. Notably, in these experiments, nitrocellulose membranes were first probed with anti-phospho-MARCKS, anti-

phospho-neurogranin, anti-phospho-GluA1, and anti-phospho-NR1 antibodies and then stripped with stripping buffer and re-probed with the anti-non-phospho-antibody. The bottom part of the same membrane was used for Western blot analysis of actin to assure that an equal amount of protein was loaded into the wells. Quantification of the immunoblots was performed by densitometric scanning of the x-ray film using a Kodak Image Station 440 CF and Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

Results

PKC activity was enhanced in prefrontal cortex after treatment with amphetamine

Elevation of PKC activity/isozymes has been found in both postmortem brain tissue and peripheral cells in BPD patients (Arnsten and Manji, 2008), and activation of PKC in the frontal cortex has been associated with manic-like cognitive changes in both rodents and non-human primates (Birnbaum et al., 2004). Basal frontal cortical PKC activity was therefore investigated in the mice that received pro-manic amphetamine treatment. Chronic treatment with the psychostimulant amphetamine significantly increased membrane PKC activity by 194.5% (Figure 1). PKC activity in cytosol fractions from the frontal cortices was also increased by 172.4%, following treatment with amphetamine (Figure 1).

PKC phosphorylation of MARCKS is enhanced in prefrontal cortex of amphetamine-treated, sleep deprived, and imipramine-treated animals, but reduced in lithium-treated animals

Phosphorylation of MARCKS in membrane fractions from frontal cortex has been used as an indicator of PKC activity in brain tissue (Bar-Am et al., 2004; Pandey et al., 2003; Yamauchi et al., 1998). MARCKS belongs to a family of proteins involved in calcium regulation and cytoskeletal restructuring. Seminal studies by Lenox and associates demonstrated that MARCKS is a target for lithium's actions (Lenox et al., 1996). The functional domain of MARCKS is bound to CaM, phosphorylated by PKC, and its activation contributes to binding of this protein to the cellular membrane.

The psychostimulant amphetamine evokes manic-like behavior in animal models at the dose used in these experiments, and sleep deprivation is also a well-established animal model for mania. We found that chronic treatment with the psychostimulant amphetamine significantly increased phospho-MARCKS (pMARCKS) levels in the membrane fraction by 197.2% (Figure 2). Nitrocellulose membranes for Western blot analysis of pMARCKS were re-probed with an anti-MARCKS antibody. The MARCKS levels in membrane fraction of frontal cortex from amphetamine-treated mice increased significantly by 151% (Figure 2). The pMARCKS and MARCKS levels in the cytosol fractions remained unchanged (Fig.2). Actin, used as a loading control for Western blot analyses for all membrane and cytosol fractions, was unaltered under these treatment conditions (Figure 2). In sleep deprived animals, we also found that pMARCKS levels were significantly increased in the membrane fraction of the prefrontal cortex up to 159% (Figure 3). The total MARCKS membrane fraction was also increased, suggesting increased translocation of MARCKS after PKC phosphorylation (Figure 3).

Antidepressant agents (particularly tricyclic antidepressant agents) can trigger manic episodes in individuals with BPD (Boerlin et al., 1998; Goldberg et al., 1999). Because the psychostimulant amphetamine and sleep deprivation enhanced pMARCKS in prefrontal cortex, we next investigated whether pMARCKS, as an indicator of PKC activation, is enhanced in prefrontal cortex of animals treated with the antidepressant imipramine. We found that pMARCKS levels were significantly increased in the membrane fraction of prefrontal cortex up to 181%, which is almost identical to the result obtained through treatment with amphetamine or sleep-deprivation (Figure 4). The total MARCKS membrane fraction was also increased, suggesting increased translocation of MARCKS after PKC phosphorylation (Figure

4). In addition, the PKC activity in membrane and cytosol fractions from prefrontal cortex of imipramine-treated animals were also significantly increased by 154% and 124% respectively, confirming the finding in phosphorylation of MARCKS (data not shown).

PKC phosphorylation of MARCKS by lithium

Lithium has been used as an anti-manic agent for more than 50 years. We thus used lithium to investigate how anti-manic agents affect PKC activity in prefrontal cortex. We found that, in animals chronically treated with lithium for four weeks, pMARCKS levels were significantly decreased in the membrane fraction from the prefrontal cortex by 33% (Figure 5). The total membrane MARCKS levels showed a non-significant trend to decrease (Figure 5).

PKC phosphorylation of presynaptic neurogranin as a target in manic related animal models

Neurogranin is a key postsynaptic protein that regulates long-term potentiation (LTP). We therefore investigated the biochemical effects of the psychostimulant amphetamine and sleepdeprivation on neurogranin levels. The pro-manic effects of these treatments are generally observed in a similar time-frame to that used in the biochemical studies. Chronic administration of the psychostimulant amphetamine or sleep deprivation in animals significantly increased levels of phosphorylated neurogranin in the frontal cortex up to 199% and 173%, respectively, as compared to control animals (Figure 6A). We also examined the phosphorylation of neurogranin in the imipramine-treated animal model. Phosphorylation of neurogranin was 189%, higher in these animals compared with the control group (Figure 6A). Total neurogranin levels in amphetamine-treated, sleep deprived, or imipramine-treated animals remained unchanged (Figure 6B).

To determine if levels of neurogranin were also altered by anti-manic agents, chronic treatment with lithium was undertaken. As predicted, and in marked contrast to the effects observed with amphetamine or imipramine, chronic lithium reduced phosphorylated levels of neurogranin in the frontal cortex by 40% (Figure 6A). Total neurogranin levels after lithium treatment remained unchanged (Figure 6B).

PKC phosphorylation of NMDA NR1 receptors as a functional regulator in pro-manic or anti-manic animal models

Recent work has shown that AMPA/NMDA receptor throughput may contribute to the pathophysiology and treatment of mood disorders (Du et al., 2004b; Zarate and Manji, 2008). Therefore, we also assessed PKC phosphorylation of NMDA receptors at the NR1S896 site, which is involved in NR1 trafficking from the endoplasmic reticulum (ER) to the membrane of functional synapses (Sanchez-Perez and Felipo, 2005). In sleep-deprived animals, phosphorylation of NR1S896 was also significantly higher (162%; Figure 7A). However, although the psychostimulant amphetamine showed a trend to increase the phosphorylation of NR1S896, this increase did not reach statistical significance. Interestingly, total NR1 levels were decreased after amphetamine treatment (Figure 7B). NMDA NR1S896 receptor phosphorylation was significantly enhanced by the antidepressant imipramine up to 128%. In contrast, chronic treatment with lithium inhibited the phosphorylation of NR1S896 by 44.4% (Figure 7A). Total NR1 changes were not significant for sleep-deprived, imipramine-treated and lithium-treated groups (Figure 7B).

PKC phosphorylation of AMPA receptors at region GluA1T840 and GluA1S831 as a molecular target in pro- and anti-manic animal models

AMPA receptors have been implicated in the treatment of BPD (Du et al., 2008; Du et al., 2004c; Du et al., 2007). We therefore investigated the phosphorylation of GluA1T840 (PKC site) and GluA1S831 (PKC /CAMKII site) in these animal models of mania. Treatment with

the psychostimulant amphetamine, the antidepressant imipramine, and sleep deprivation all significantly enhanced phosphorylation of GluA1 at T840 up to 132%, 129%, and 122%, respectively (Figure 8A). In contrast, levels of AMPA receptor GluA1T840 (a PKC site) were decreased in lithium-treated animals by 38.4% (Figure 8A). Similarly, GluA1S831 (a CAMKII/PKC site) was significantly increased in amphetamine-treated, and imipramine-treated animals to 168% and 147%, respectively (Figure 8B). Consistent with previous study (Vyazovskiy et al., 2008), phosphorylation of GluA1S831 had a very strong trend to increase by 150% in sleep-deprived animals (N=8 for each group, student's t test, p=0.08, Figure 8B). However, lithium treatment did not significantly affect on GluA1S831, which is also consistent with previous finding (Du et al., 2004a). Changes in total GluA1 levels were not significant in any of the experimental groups (Figure 8C).

Discussion

Accumulating clinical, preclinical, and genetic data have implicated overactive PKC intracellular signaling in mania (Einat et al., 2004). However, the functional targets of PKC have not been identified. We therefore undertook this series of studies to ascertain whether PKC modulation of these critical substrates is involved in the pathophysiology and treatment of manic-like behaviors. We found that 1) PKC activity in the prefrontal cortex was enhanced by treatment with the psychostimulant amphetamine; 2) phosphorylation of MARCKS, another indicator for PKC activity in vivo, was increased in the prefrontal cortex in two animal models of mania: amphetamine treatment and sleep deprivation; MARCKS phosphorylation was decreased in animals treated with the anti-manic agent lithium; 3) phosphorylation of neurogranin, a selective postsynaptic PKC molecular target, was similarly increased in these two animal models of mania and reduced after treatment with lithium; and 4) phosphorylation of the NMDA receptor NR1S896 and GluA1T840 was enhanced in sleep deprived animals as well as those treated with the antidepressant imipramine; treatment with lithium reduced the phosphorylation.

PKC is implicated in the pathophysiology of BPD in humans

Previous studies (Wang and Friedman, 1996) reported increased PKC activity and translocation in postmortem brains of individuals diagnosed with BPD compared to controls; these effects were accompanied by elevated levels of selected PKC isozymes in the cortex of BPD subjects. It has also been shown that mood stabilizers decreased PKC in the platelets of patients with BPD (Pandey et al., 2007). Moreover, recent human genetic evidence has also identified polymorphisms that confer susceptibility to BPD in the gene encoding for DGKH (Baum et al., 2008), which regulates the formation of diacyl glycerol (an activator of PKC), IP3, and phospholipase C (the initial enzyme in the PKC cascade) (Turecki et al., 1998). In addition, a recent preclinical study from our group found that tamoxifen (a non-steroidal anti-estrogen and known PKC inhibitor (Baltuch et al., 1993)) significantly reduced amphetamine-induced hyperactivity in a large open field and attenuated amphetamine-induced phosphorylation of GAP-43 at the PKC site (Einat et al., 2004; Einat et al., 2007). Three independent clinical trials also found that tamoxifen significantly improved the symptoms of mania compared to placebo (Bebchuk et al., 2000; Yildiz et al., 2008; Zarate et al., 2007).

Overactive PKC signaling in the prefrontal cortex may explain many of the symptoms of mania (Arnsten and Manji, 2008). Functional imaging studies have shown reduced activity in the right prefrontal cortex during mania (Blumberg et al., 2003; Blumberg et al., 1999). In addition, structural imaging studies have further shown a loss of prefrontal volume in untreated patients with BPD (Blumberg et al., 2006).

Antidepressant agents (particularly tricyclic antidepressant agents) can trigger manic episodes in individuals with BPD (Boerlin et al., 1998; Goldberg et al., 1999). However, whether or not

PKC activity is decreased in patients with depression or in animal models of depression remains unclear. Pandey and colleagues found that MARCKS phosphorylation was significantly decreased in the membrane fraction of prefrontal cortex and hippocampus obtained from both depressed and non-depressed suicide subjects (Pandey et al., 2003; Pandey et al., 2004). However, in another study, PKC activity was increased in the platelets from drug-free patients with depression (Pandey et al., 1998). It is thus possible that this enhancement in PKC activity may contribute to the pro-manic effects of imipramine.

PKC is a target for the anti-manic agents lithium and VPA

Consistent evidence exists that lithium, at therapeutically relevant concentrations, exerts major effects on the PKC signaling cascade. Studies in rodents have demonstrated that chronic (but not acute) lithium treatment produces an isozyme-selective reduction in PKC α and ϵ in the frontal cortex and hippocampus (Chen et al., 2000b; Li et al., 1993; Manji et al., 1993; Manji and Lenox, 1999). Furthermore, chronic lithium treatment has been shown to dramatically reduce the hippocampal levels of the protein MARCKS, which is one of the PKC substrates implicated in regulating long-term neuroplastic events (Lenox et al., 1992). Notably, the anti-manic agent VPA, which is structurally dissimilar to lithium, produces very similar effects to those of lithium on PKC α and ϵ isozymes and on MARCKS, one of the PKC substrates (Chen et al., 1994; Lenox and Hahn, 2000; Manji and Chen, 2000; Manji and Lenox, 1999, 2000; Watson et al., 1998).

PKC activity in animal models of mania

In this study, we used two animal models of mania. Several independent laboratories have demonstrated that both acute and chronic amphetamine treatment alter PKC activity, PKC's relative cytosol to membrane distribution, and phosphorylation of GAP-43, which has been implicated in long-term alterations of presynaptic neurotransmitter release (Giambalvo, 1992a,b; Gnegy, 1993; Iwata et al., 1997). Sleep deprivation is another animal model of mania, and its effects can be prevented by lithium (Gessa et al., 1995). In synaptoneurosomes of rat cortex and hippocampus, GluA1-containing AMPA receptor levels and phosphorylation of GluA1S845 are high during wakefulness and low during sleep, suggesting a regulation of glutamate receptor function through phosphorylation (Vyazovskiy et al., 2008). In addition, in that study the sleep deprived group showed a nearly 50% increase in total GluA1 levels in synaptoneurosomes compared with the waking group (Vyazovskiy et al., 2008). The present study focused on the PKC targets that regulate synaptic plasticity and synaptic transmission. We found that PKC-phosphorylation of three important synaptic targets—phospho-neurogranin, phospho-NMDA, and phospho-AMPA—were related to manic behavior in four pro- and anti-manic animal models. Taken together, the evidence suggests that PKC phosphorylation may contribute to the pathophysiology of mania and the formation of manic behaviors.

The PKC target neurogranin is an important molecule regulating synaptic function

Neurogranin is a calcium-sensitive CaM-binding protein, whose CaM-binding affinity is attenuated by oxidation and phosphorylation by PKC (Giambalvo and Price, 2003; Huang et al., 2000). Binding of neurogranin to CaM can be restored by dephosphorylation of neurogranin with protein phosphatase I (PP1), protein phosphatase 2A (PP2A), and by the calcium-CaM dependent protein phosphatase 2B (Li et al., 2003). It is not only a major PKC target, but also an upstream regulator of three major signalling cascades—calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), and PKC—that are thought to play important roles in the pathophysiology and treatment of severe mood disorders (Barbiero et al., 2007; Coyle and Duman, 2003; Manji and Chen, 2000; Popoli et al., 2001). Under manic conditions, increased phosphorylation of neurogranin by PKC (Figure 3) liberates calmodulin

towards activation of CAMKII and CAMKIV, which is the key for the formation of LTP. However, we postulate that lithium treatment increases the binding of neurogranin with calmodulin and leads to lower activation of CaMKII.

Regulation of the NMDA receptor NR1S896 is related to the trafficking of NR1 to the synapses

Accumulating data have shown that the glutamatergic system is involved in the pathophysiology and treatment of mood disorders (Maeng and Zarate, 2007; Zarate et al., 2006). The NMDA receptor is present in the brain primarily as a tetramer, in which two subunits are obligatory glycine-binding NR1 subunits, while the remaining two are typically glutamate-binding NR2 subunits. NMDA receptors are targets for PKC and PKC-dependent tyrosine kinase cascades. Direct phosphorylation of NMDA receptors by PKC increases the sensitivity of the NMDA receptor to inactivation by intracellular calcium. In the hippocampus, PKC is a point of convergence for a variety of G-protein coupled and growth factor receptors that maintain control over NMDA receptor function and synaptic plasticity (McDonald et al., 2001). The phosphorylation of NR1 at S896 has been reported to be critical for NR1 receptor transportation to the synapses (Sanchez-Perez and Felipo, 2005). Therefore, enhanced NR1S896 phosphorylation in the prefrontal cortex would lead to the efficient transportation of NR1 to the synapses in this brain region.

AMPA GluA1T840 phosphorylation is significantly increased in animals treated with imipramine and sleep deprived animals, and decreased in animals treated with lithium

The AMPA subtypes of glutamate receptors appear to play a major role in regulating short- and long-term forms of synaptic plasticity (Lee, 2006). Regulation of GluA1 trafficking via phosphorylation of its PKA site (S845) has been shown to be important for the treatment of manic-like behaviors (Du et al., 2008; Du et al., 2004a). Notably, this trafficking is now known to depend, in large part, upon AMPA receptor subunit phosphorylation at serine, threonine, or tyrosine residues located on the intracellular C-terminal domain (reviewed in (Du et al., 2004a). Protein kinase signaling cascades, such as PKA, PKC, CaMKII, and tyrosine kinases (Trks; or Src Family Kinases) are involved in site-specific AMPA receptor phosphorylation (Banke et al., 2000; Barria et al., 1997; Boehm et al., 2006; Roche et al., 1996). Other glutamate (NMDA receptors) and G-protein coupled receptors (mGluRs, Dopamine Receptors DA1, serotonin receptors) can also regulate AMPA receptors through protein phosphorylation and/or dephosphorylation mechanisms (Du et al., 2004a; Zarate et al., 2003). A threonine-840 (T840) on the GluA1 subunit of AMPA receptors was identified as a novel phosphorylation site by PKC in vitro and is functionally relevant to synaptic plasticity (Lee et al., 2007). Here, we identified GluA1T840 as a target of PKC, conferring its effects on mood-associated behaviors (Figure 7).

The animal models of mania used here—amphetamine-treated and sleep-deprived animals—are animal models of acute mania. During the occurrence of acute manic-like behaviors, we propose that there is increased neuronal activity. The phosphorylation of MARCKS, neurogranin, NMDA, and GluA1 should result in increased neuroplasticity, which may contribute to the pathophysiology of the manic episode.

In summary, biochemical data presented here indicate that abnormalities in the frontal cortical PKC signaling cascade may play an important role in the pathophysiology and treatment of BPD. Accumulating preclinical and clinical data suggest that PKC inhibitors may be potential therapies for BPD (Einat et al., 2007; Yildiz et al., 2008; Zarate et al., 2007). This work elucidates the mechanisms whereby PKC activation triggers and amplifies the functional cascade of many target proteins—including NMDA receptors, AMPA receptors, neurogranin, and MARCKS—to regulate pre- and post-synaptic functions in prefrontal cortex. These results

suggest that modulation of either PKC or its functional targets may lead to the development of novel therapeutics for mood disorders.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institute of Mental Health, National Institutes of Health, and by NIMH (P20 MH077967 to CC) and NIH Director's Pioneer award (to GT). We thank Ioline Henter for outstanding editorial assistance.

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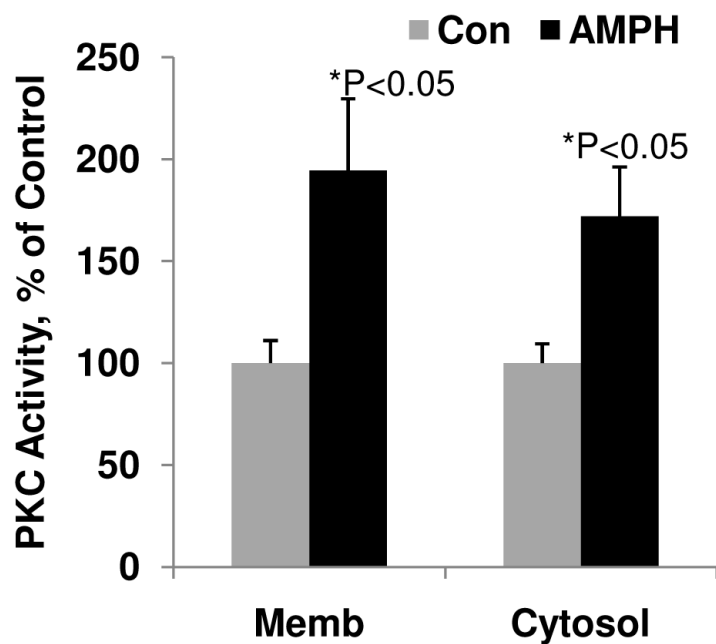


Figure 1. The psychostimulant amphetamine enhanced PKC activity in both membrane fraction and cytosol fraction of forebrain in vivo

Mice were treated with amphetamine (AMPH, 5mg/kg, i.p.) for 10 days. Prefrontal cortex (Fcx) was dissected and fractionated into membranous and cytosolic fractions. PKC activity was determined with neurogranin (Ng) peptide as a substrate. (Student's *t*-test, * $p < 0.05$, $N = 5-8$).

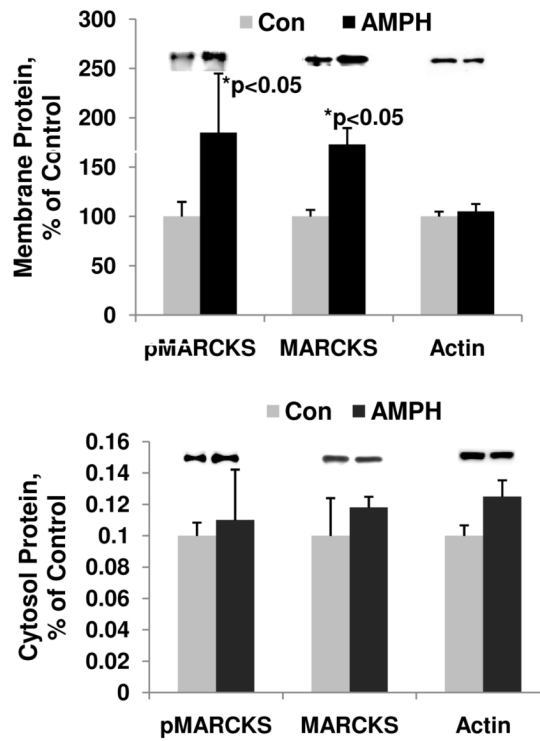


Figure 2. Phosphorylation of MARCKS at a PKC site in membranous fraction from the forebrain of amphetamine-treated animals

(A) pMARCKS, MARCKS, and actin levels in membrane fraction from the forebrain of amphetamine (AMPH)-treated animals.

(B) pMARCKS, MARCKS, and actin levels in cytosol fraction from the forebrain of amphetamine (AMPH)-treated animals.

Data are presented as mean \pm SE (Student's *t*-test, * $p < 0.05$, N=4-8).

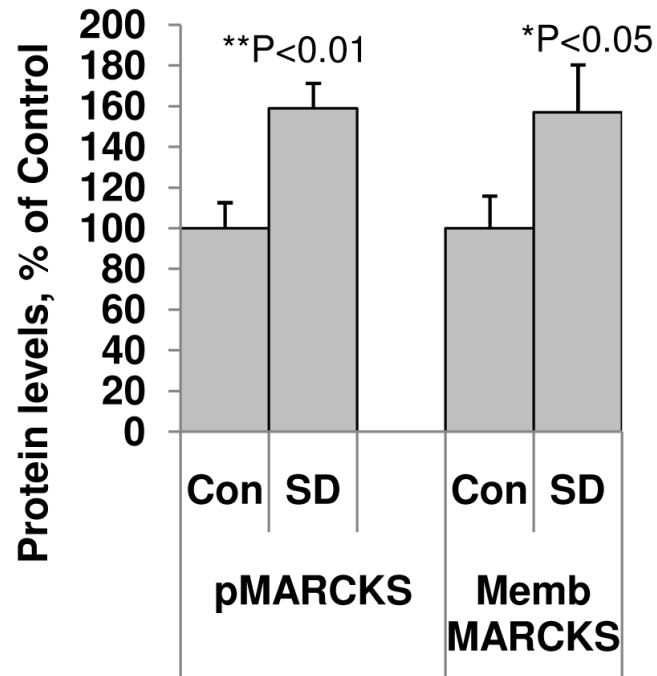


Figure 3. PKC phosphorylation of MARCKS in a sleepdeprivation (SD) animal model of mania Western blot analysis was performed to determine the pMARCKS or MARCKS in the membrane fractions. (N=8 animals per group, Student's *t*-test, * $p<0.05$) Data are presented as mean \pm SE.

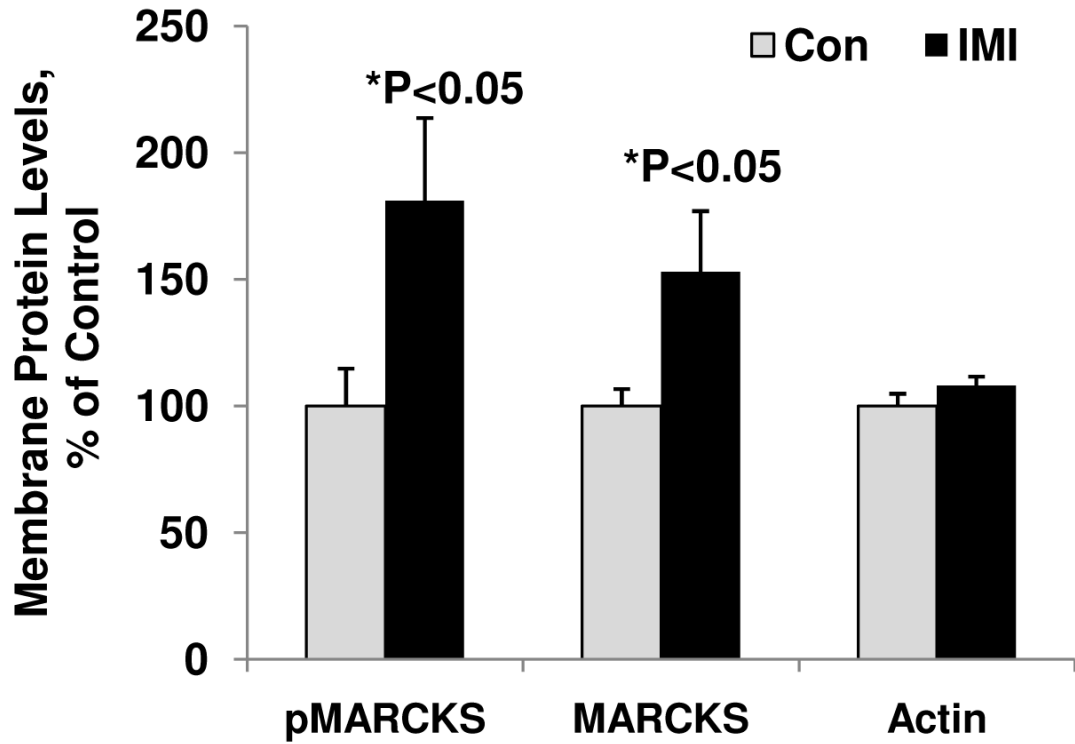


Figure 4. The antidepressant imipramine significantly enhanced pMARCKS in membrane fraction in prefrontal cortex

Western blot analysis was performed to determine the pMARCKS or MARCKS in the membrane fractions (N=4-8 animals per group, Student's *t*-test, * $p < 0.05$). Data are presented as mean \pm SE.

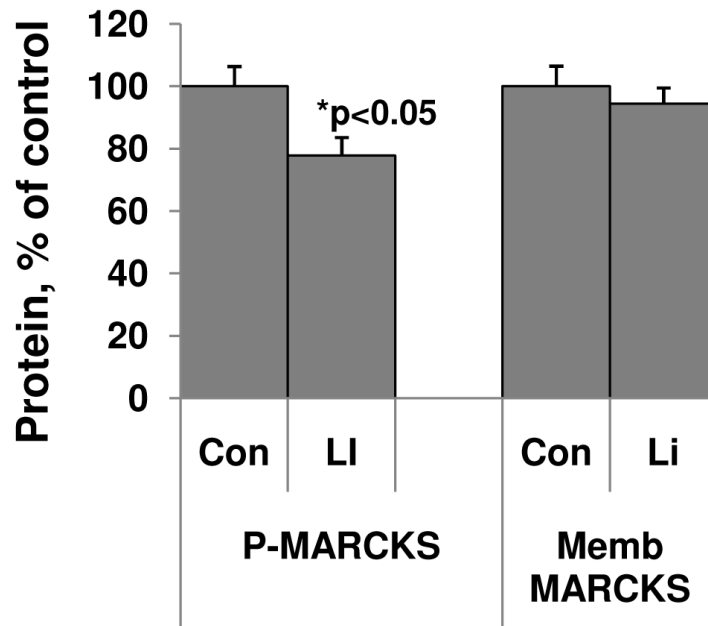


Figure 5. Lithium (Li) significantly attenuated PKC phosphorylation of MARCKS
C57/B6 mice were treated with lithium for four weeks. Western blot analysis was performed to determine the pMARCKS and MARCKS in the membrane fractions (N=14, Student's *t*-test, * $p < 0.05$). Data are presented as mean \pm SE.

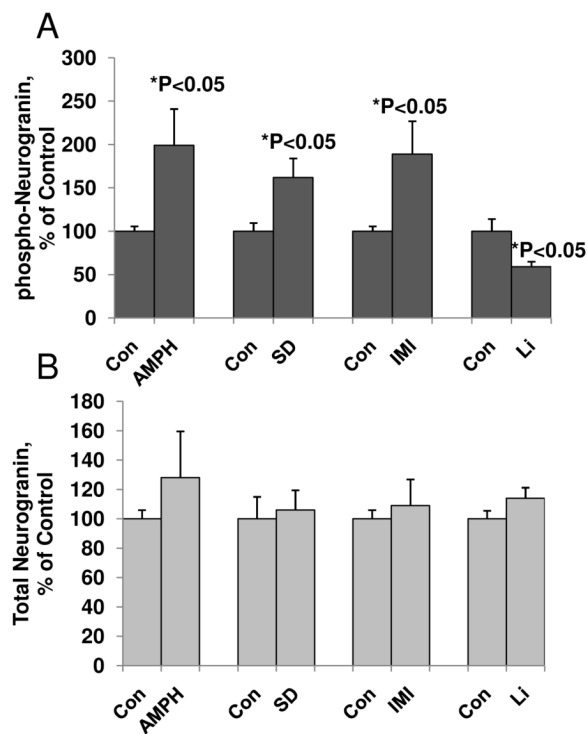


Figure 6. The presynaptic protein neurogranin is a target of PKC in pro- and anti-manic animal models

(A) Phosphorylation of neurogranin of prefrontal cortex from mania related animal models. Western-blot analysis of phosphorylated and non-phosphorylated neurogranin levels in crude homogenates from the frontal cortex of animals undergoing sustained treatment with amphetamine (AMPH), sleep deprivation (SD), imipramine (IMI), and lithium (Li). (B) Lack of effect on total neurogranin levels in the frontal cortex in all animal models. For all experimental groups, N=6-8, Student's *t*-test, **p*<0.05. Data are presented as mean±SE.

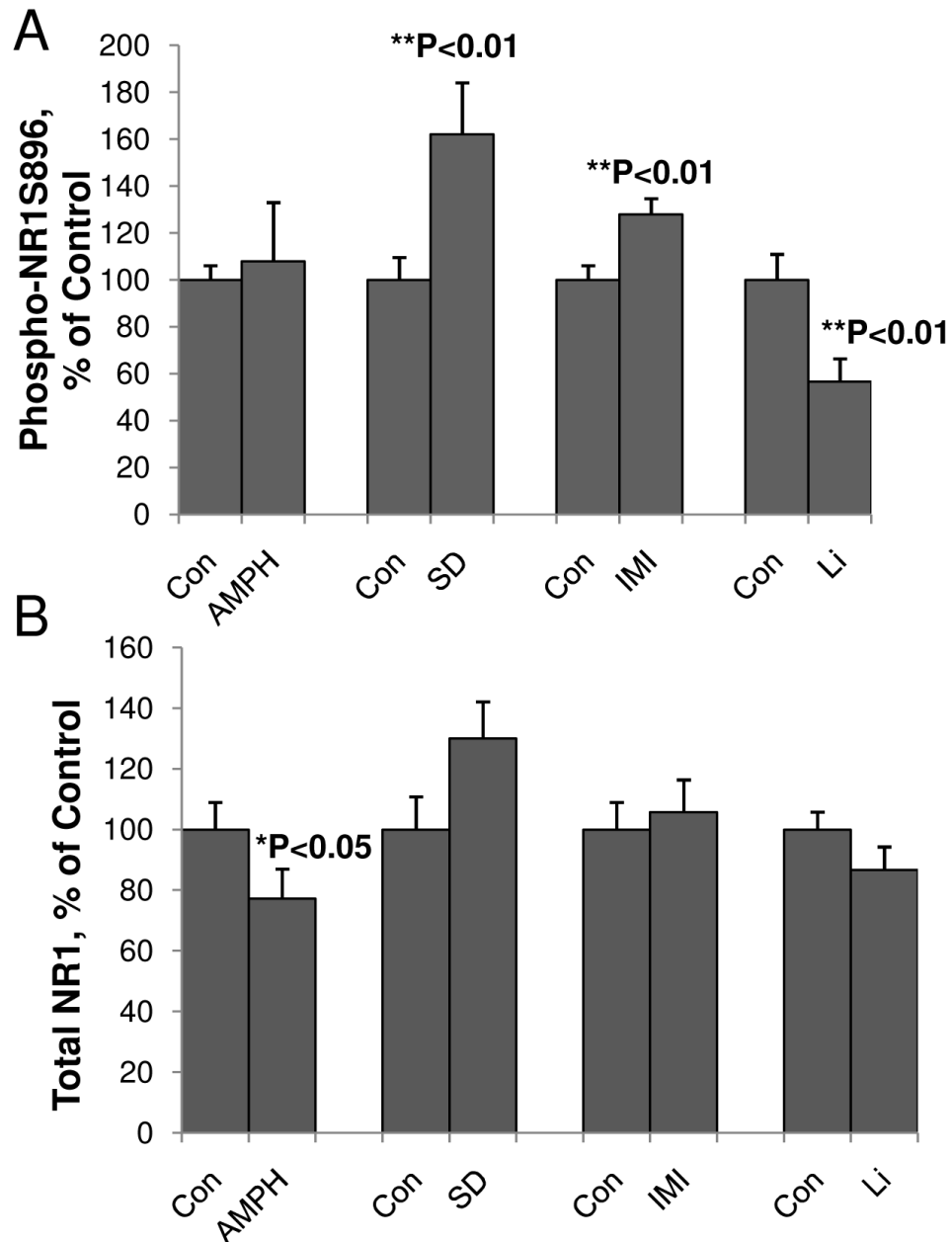


Figure 7. Phosphorylation of NMDA receptor NR1S896 at PKC site in mania-associated animal models

Total protein samples from the prefrontal cortex of amphetamine (AMPH)-, imipramine (IMI)-, or lithium (Li)- treated animals or sleep-deprived (SD) animals were subjected to Western blot analysis with anti-phospho-NR1S896 or anti-NR1 antibodies. Data are presented as mean \pm SE (** p <0.01 Student's t -test. N =8-20 for each group).

(A) Phospho-NR1S896 levels in mania related animal models..

(B) Total NR-1 levels in mania related animal models..

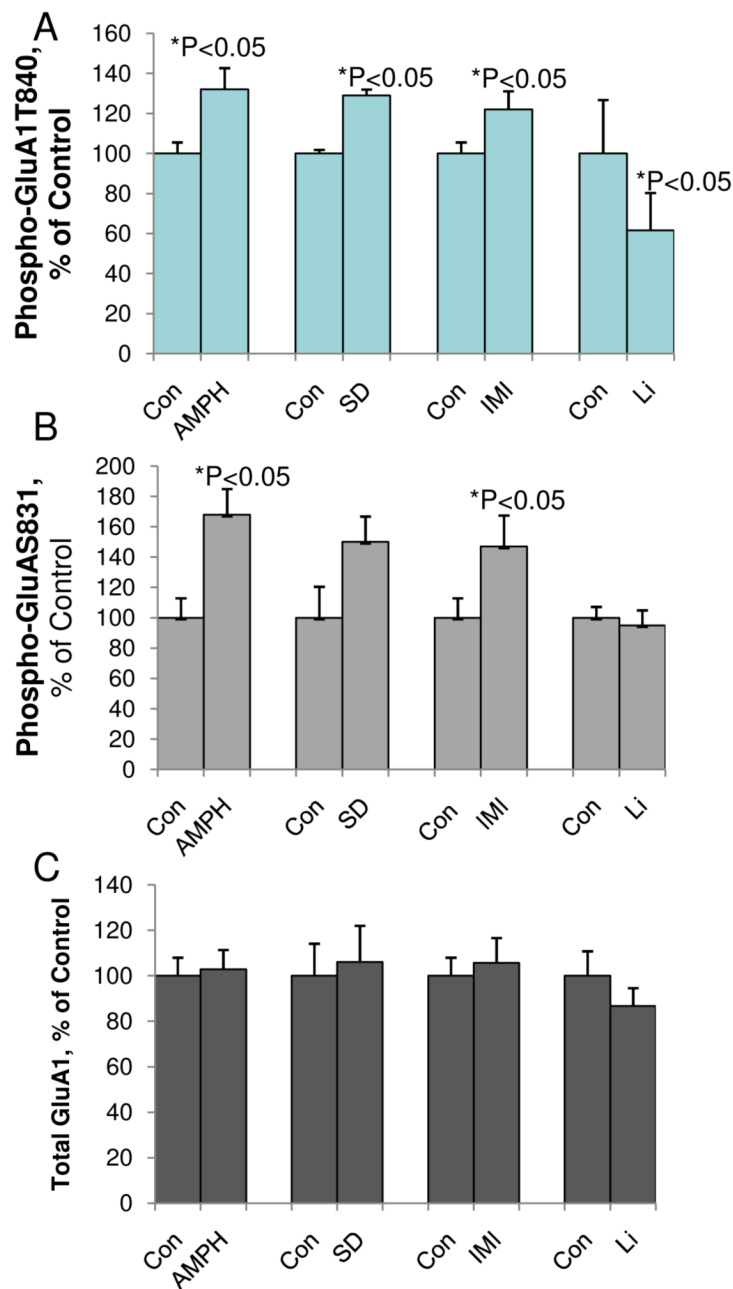


Figure 8. AMPA GluA1T840 and GluA5831 phosphorylation regulated by PKC in mania-associated animal models

Total protein samples from the prefrontal cortex of sleep deprived animals or amphetamine (AMPH)-, imipramine (IMI)-, or lithium (Li)-treated animals were subjected to Western blot analysis with anti-phospho-GluA1T840, anti-phospho-GluA1S831, or anti-GluA1 antibodies. Data are presented as mean \pm SE (* p <0.05 Student's t -test. $N=8$ animals for each group).

(A) Phospho-GluA1T840 levels in mania-related animal models.

(B) Phospho-GluA1S831 levels in mania related animal models

(C) Total GluA1 levels in mania related animal models.