Hyper-activated protein kinase C mediates the reduced AMPA receptor surface expression in prenatal cocaine exposed brains: the role for diacylglycerol, diacylglycerol kinase, and 3-phosphoinositide-dependent kinases.

By

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Abstract

Hyper-activated protein kinase C mediates the reduced AMPA receptor surface expression in prenatal cocaine exposed brains: the role for diacylglycerol, diacylglycerol kinase, and 3-phosphoinositide-dependent kinases.

By

Jingjing Liu

Advisor: Professor Hoau-Yan Wang

Prenatal cocaine exposure induced neurobehavioral and synaptic changes are in part mediated by the defected alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamatergic receptor (AMPAR) neurotransmission. This abnormality of AMPAR system is related to reduced AMPA-GluR2/3 synaptic targeting, which is resulting from sustained phosphorylation of glutamate receptor-interacting protein (GRIP) by hyper-activated protein kinase C (PKC) (Bakshi *et al.*, 2009). The underlying molecular mechanism responsible for PKC hyper-activation, however, remains obscure. Blockade of PKCg and PKC/Mz by their specific pseudosubstrate inhibitors restores AMPAR synaptic targeting, demonstrating that PKC is essential in producing AMPAR abnormalities in prenatal cocaine exposed animal brains. The enhanced PKC activation by prenatal cocaine exposure correlates with an elevated 3-phosphoinositide-dependent protein kinase-1 (PDK1) level, and a persistent increase of synaptic membranous diacylglycerol (DAG) level resulting from down-regulated GRIP-associated DAG kinase (DGKγ and DGKζ) subtypes. Altogether, these data provide the molecular underpinning

for persistent PKC activation in prenatal cocaine-exposed brain and suggest that suppression of PKCγ and PKC/PKMζ can restore GluR2/3 synaptic targeting and AMPAR function. Importantly, the data derived from this study may provide a novel strategy to treat neurobehavioral abnormalities resulting from prenatal cocaine exposure.

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Chapter 1. Introduction

What is prenatal cocaine exposure? What does it cause in human offspring and animal models?

Cocaine is a highly addictive CNS stimulant. The abuse of cocaine among women who are in their childbearing age has been becoming an increasingly serious problem worldwide. Cocaine is a very small molecule. It readily crosses the placenta and the blood-brain barrier of the fetus. Therefore it could easily affect the offspring.

Gestational cocaine exposure causes enduring cognitive deficits in offspring of both human and animal models. It was observed in human prenatal cocaine is related to premature birth, low birth weight, and multiple birth defects. Prenatally cocaine exposed adolescents as well as adults show vulnerability to stressors and learning disability in their later lives. (Dow-Edwards *et al.*, 1999; Thompson *et al.*, 2005; Morrow *et al.*, 2006; Salas-Ramirez *et al.*, 2010). Neurochemical and morphological changes were observed in animal models of prenatal cocaine exposure, such as uncoupling of Gs protein from D1 dopamine receptors (Wang et al., 1995; Friedman et al., 1996; Jones et al., 2000). Cognitive deficits such as deficits with attention, motor skill, and learning were also observed in animals exposed to cocaine prenatally (Romano and Harvey, 1996; Harvey et al., 2001; Salas-Ramirez *et al.,* 2010).

What does cocaine do in CNS? How does cocaine modify glutamate system in the brain?

Drug addiction, including cocaine addiction, has long been thought to involve learning and plasticity changes in the mesolimbic-cortical system, which is enriched with both dopaminergic and glutamatergic innervations. The effect of cocaine in CNS has been intensively studies in the

past decades, particularly the role of cocaine in brain reward circuit. Cocaine blocks dopamine reuptake inhibitor and increases dopamine level in the brain mesolimbic-cortical system, which contribute to the euphoria of cocaine use. In brain reward circuit the ventral tegmental area (VTA) sends dopaminergic projections into frontal lobe, nucleus accumbens (NAc) and hippocampus (HP). VTA receives excitatory glutamatergic projections back from almost all the areas it projects to expect NAc (Figure 1). It has been reported that dopamine neuron firing and dopamine level are regulated by glutamate projections. Besides the dopamine system, it has been long established that cocaine modifies glutamate transmission system in the brain as well (REF??). Glutamate transmission undergoes enduring neuroplasticity in the brain. Lots of evidence shows that cocaine induces changes in proteins that regulate pre- and postsynaptic glutamate neurotransmission. Therefore glutamatergic transmission plays a crucial role in cocaine abuse induced changes in brain. Among various glutamatergic receptor systems, the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamatergic receptor (AMPAR) appears to be particularly sensitive to cocaine in adult rodent brain. Single or multiple dosages of cocaine both modifies the AMPA/NMDA ratio in dopamine neurons without changing the expression level of AMPAR/NMDAs representing synaptic enhancement. The increased AMPA/NMDA ratio is due to replacement of lower conductance CI-AMPARs with higher conductance CP-AMPAR in VTA suggesting the subunits redistribution of AMPAR caused by drug administration (Ungless *et al.*, 2001; Saal *et al.*, 2003; Borgland *et al.*, 2004; Boudreau & Wolf, 2005; Wolf & Tseng, 2012). Cocaine exposure was also reported has long term effect on activation of CP-AMPARs in the NAc. Meanwhile AMPAR subunits GluR1 and GluR2 knockdown studies have shown to attenuate brain stimulation, food and cocaine reward (Dong *et al.,* 2004; Mead *et al.,* 2005).

What does prenatal cocaine do in CNS? How does prenatal cocaine modify glutamate system?

Even though the underneath mechanism of prenatal cocaine effect has not been fully understood, glutamatergic neurotransmission, the most prominent excitatory neuronal transmission pathway in the brain, is reported vulnerable to modifications by *in utero* cocaine exposure in animal models (Bakshi *et al.*, 2009; Bakshi *et al.*, 2011; Bellone *et al.*, 2011). Prenatal cocaine decreases AMPAR subunit GluR2 expression on the synaptic membrane. The switching of AMPA subunit GluR2-lacking receptors into GluR2-containing receptors was delayed in the VTA which implies a delayed maturation of glutamatergic transmission in brain reward system (Bellone *et al.*, 2011). Prenatal cocaine increases dendritic spine density in multiple regions in the brain (Salas-Ramirez et al., 2010; Frankfurt M *et al.,* 2009; Morrow BA et al., 2007). Interestingly, it's been found that the abundance of postsynaptic AMPAR subunit GluR2 correlates with the density and size of spine and number of functional synapses. Overexpression of GluR2 subunit of AMPAR increases spine size and density in HP neurons, and induces spine formation in GABA-releasing interneurons that normally lack spins, while knockdown of endogenous GluR2 expression reduces spine number. The ability to increase spine number and size seems to be specific for GluR2. The N-terminal domain (NTD) of GluR2 is the specific site for an extracellular protein-protein interaction that is important for the formation, growth and maintenance of dendritic spines (Passafaro et al., 2003). The increased spine number is associated with an increase in EPSC frequency, indicating that this mechanism also increases the number of functional synapses. The increase of spine and synapse number appears to be entirely mediated by the N terminal of GluR2 (Passafaro et al., 2003; Saglietti et al., 2007). Moreover, GluR2, specifically, insertion into the synaptic membrane during early development has been identified regulating

dendritic spine morphology and function which are driven by synaptic activity, long-term potentiation, and possibly learning and memories (Engert & Bonhoeffer, 1999; Matsuzaki *et al.*, 2001; Kumar *et al.*, 2002; Passafaro *et al.*, 2003), implying the molecular mechanism of distribution and targeting of synaptic membranous GluR2 is crucial in understanding the early formation of addiction and postnatal tendency towards addiction after prenatal exposure to drugs. Therefore, how prenatal cocaine modify AMPAR particularly GluR2 becomes a major interest of our research.

AMPA receptor and its adaptor proteins

AMPA receptors are cation channels composed of different combinations of GluR1/2/3/4 subunits (Hollmann & Heinemann, 1994; Roche et al., 1994; Wenthold et al., 1996) (Figure 2). The proteins of subunits GluR1/2/3 are expressed increasingly during postnatal development and they mature and stabilize by postnatal day 20 (Bettler et al., 1990; Hollmann &Heinemann, 1994; Zhu et al., 2000). In the brain, GluR1/GluR2 containing receptors form the regulated pathway whereas GluR2/GluR3 containing AMPARs continuously cycle between the postsynaptic membrane and intracellular compartments form the constitutive pathway (Malinow et al., 2000). The subunits of AMPARs are going under recycles. The synaptic targeting and translocation of GluRs are achieved through the interaction of their C-terminus tails with the postsynaptic density 95/Discs large/zona occludens-1 (PDZ) domain containing protein GRIP (Glutamate Receptor Interacting Protein) (Dong et al., 1997; Chung et al., 2000; Kim et al., 2001). During early development, expression levels of GRIP1 and GRIP2 stabilize around postnatal day 8 and 14 respectively (Dong et al., 1999). AMPARs mediate fast synaptic transmission. Different subtypes have functional difference and determine single channel

conductance and postsynaptic excitability. Therefore, regulation of different AMPARs expression, trafficking and turnover are crucial to early establishment of synaptic plasticity and neuronal development.

Moreover, GluR2, specifically, is the subunit that governs calcium permeability of AMPAR. GluR2-containing AMPAR is calcium impermeable with lower single channel conductance while CluR2-lacking AMPAR is calcium permeable with higher single channel conductance (Figure 2). GluR2's insertion into the synaptic membrane during early development regulates dendritic spine morphology and function which are driven by synaptic activity such as long-term potentiation and possibility learning and memories (Engert & Bonhoeffer, 1999; Matsuzaki *et al.*, 2001; Kumar *et al.*, 2002; Passafaro *et al.*, 2003). GluR2 is associated with many adaptor proteins including membrane fusion protein NSF, scaffolding protein GRIP1/ABP (GRIP2), and PICK1 (protein interacting with C kinese-1) (Figure 2). They maintain synaptic accumulation of the receptors possibly by limiting endocytosis. The synaptic insertion of GluR2-containing AMPARs mediated by GRIP influences the electrophysiological property of synapses and AMPAR transmission. We confirmed in previous experiment that although prenatal cocaine exposure didn't change the expression and association of NSF and PICK1 with GluR2, a severe reduction in the association of GRIP1/2 with GluR2 was noted (Bakshi et al., 2009). This uncoupling of GluR2 from GRIP1/2 is caused by increased phosphorylation of GRIP by PKC (Bakshi *et al.*, 2009). Correspondingly, our lab found that AMPA mediated long-term depression (LTD) was dramatically reduced in prenatally cocaine exposed adolescent rat brain. This hyper PKC activity also promotes F-actin levels and increases dendritic length and density considered a compensating mechanism for prenatal cocaine produced altered synaptic chemical transmission (Schubert *et al.*, 2006; Bakshi *et al.*, 2011). Acute systemic injection of cocaine promotes

increased rapid phosphorylation on PKC activation loop at multiple brain areas which is a critical step for PKC activation process (Xue *et al.*, 2012). All those findings imply that PKC plays a crucial role in mediating prenatal cocaine effect on arrested development of AMPAR transmission through phosphorylation on GRIP. AMPAR medicated long-term potentiation LTP and LTD are widely believed the cellular basis of learning, memory and even drug addiction including cocaine (reward driving learning) (Kauer & Malenka, 2007). PKC is also widely believed involving in cocaine-induced memories in brain rewarding system (Lai *et al.*, 2008; Li *et al.*, 2011). Inhibition of PKC cytosol-to-membrane translocation without overt effects on enzyme activity is effective in attenuating some long-lasting prenatal cocaine-induced neurobehavioral abnormalities (Wang et al., 1999; Hahn et al., 2005). Therefore, we focused on the prenatal cocaine effects on PKC and how it modifies AMPAR and its scaffolding protein GRIP at early step of development of animal brains. We believe that elucidating the underlying molecular mechanism can improve our understanding of the altered cognitive, social behavior, and relation to drug addiction in human offspring with maternal cocaine exposure and provides pharmacological value. We hypothesized that prenatal cocaine exposure modifies AMPAR transmission by promoting PKC hyperactivation and hyperphosphorylation of GRIP.

Protein kinase C and its activation by DAG, DGK, and PDK1

PKC is family of serine/threonine kinases that are typically activated by diacylglycerol (DAG) that is formed by hydrolysis of the membrane phospholipid by PLC. PKC isoforms undergo a series of phosphorylation events to mature into the catalytically-competent form (Figure 3). All PKCs' maturation is initiated by phosphorylation at PKC's activation loop threonine by the upstream kinase PDK1, and then requires phosphorylation at the hydrophobic and turn motifs.

By then PKC still states inactive with the inhibitory pseudosubstrate peptide blocking its active site. DAG-mediated membrane binding provides the energy required to expel the inhibitory peptide from the active site and allow PKC to phosphorylate downstream substrates.

Conventional PKC and novel PKC isoforms contain DAG-sensitive C1 domains (cysteine-rich domain) for DAG binding. Even though atypical PKCs, such as PKC/PKMζ, do not require DAG for activation, their translocation and duration of being tethered on the membrane is positively correlated with DAG in the membrane (Asaoka *et al.*, 1992; Huang & Huang, 1993; Ron & Kazanietz, 1999). Since PKC can activate Src tyrosine kinase (Ali and Salter, 2001) and protein kinases are often targeted to close proximity of their substrates ensuring that they phosphorylate only the proper targets and prevents inappropriate phosphorylation events (Pawson and Nash, 2000; Smith and Scott, 2002), it leaded us to hypothesize that subset of PKC isozymes are associated with GRIP (the substrate).

DAG level in synaptic membrane is under the regulation of diacylglycerol kinase (DGK), a kinase that metabolizes DAG to yield phosphatidic acid (PA) (Figure 4). The DAG mass contained in cell membrane can be measured by utilizing standard E. coli DAG kinas, in the presence of radioactive ATP. DGK phosphorylates the substrate DAG to yield [γ-32P] phosphatidic acid (PA), which could then be separated by thin-layer chromatography (TLC) for radioactivity assay.

DGK is a key determinant of DAG. Normally DAG signaling at synapses is very transit. Increased DAG level is wiped out by down regulator DGK in a fast manner to terminate DAG downstream signaling. Observed prolonged life time of DAG at synaptic membrane in none-stimulated tissue of prenatally cocaine exposed animals suggests a lost regulation from DGK. So far, ten mammalian isozymes have been identified (Figure 4), and are classified into five

subgroups based on their primary structure. DGK isozymes differ from each other mostly by their primary structure and tissue distribution. DGKs are a well-conserved family of protein among most species, such as drosophila melanogaster, caenorhabditis elegans, mammals, and plants. All mammalian DGK isozymes have: (1) A conserved catalytic domain, which contains amino acid segments that are identical in all DGKs including the ATP-binding site; (2) The regulatory domain including at least two cysteine-rich regions (CRDs) that are predicted to bind DAG so as to localize DGKs where DAG accumulates in the membrane, as well as to bind DNA, lipid, and protein. Besides the common structures, (1) Type I DGKs have a pair of Ca2+-binding EF-hands; (2) Type II DGKs have a pleckstrin homology(PH) domain at their N-terminus, and the catalytic domain is split into two domains; (3) Type III, DGKε, has the simplest structure; (4) Type IV, DGKζ and ι, have a myristoylated alanine-rich C kinase substrate(MARCKS) homology domain that overlaps with a nuclear localization signal and four ankyrin repeats; (5) Type V, DGKθ, has three CRD domains, a PH domain in the middle of the protein that overlaps with a so-called Ras-associating domain(RA), and a proline-rich region at its extreme N-terminus. The structures are shown in Figure 4. Most DGK isozymes show high expression in the brain, often in distinct brain regions, suggesting that each individual isozyme has a unique function. The distribution of DGKs is identified by mRNAs in situ hybridization and immunocytochemistry in rat brain and is concluded in Table 1. DGK-β, γ and ζ are abundantly present in the olfactory bulb and the hippocampus, and these brain regions are known to show synaptic plasticity and are related to learning and memory. It has been reported that a few PKC isozymes co-localize with some DGK isozymes and phosphorylate and regulate them. Translocation to membranes is believed to be part of the activation mechanism of DGKs in General. Like PKC, DGK activation requires cytosol-to-membrane translocation which is

reported in responses to DAG and PKC (Jurgen van Baal *et al.,* 2005). DGKζ is found co-localized with PKC in a signaling complex (Bai Luo *et al.,* 2003). We identified the PKC isozymes that are co-localized with GRIP in the signaling complex. Therefore, we intend to identify the DGK isozymes that are co-localized with GRIP, which are related to the hyper-activation of PKCs.

All types of PKCs require phosphorylation on the T-loop (activation loop) residue of the kinase domain by the upstream kinase PDK1 to be activated (Chou *et al.*, 1998; Le Good *et al.*, 1998; Balendran *et al.*, 2000).Therefore synaptic DAG, DGK, and PDK1 expression and/or activities are regulators and could be indicators of PKC activation. Also, PKC pseudo-substrate inhibitor peptides selectively inhibit PKCs via binding to the pseudo-substrate region in the regulatory domain without affecting the distribution of PKC, providing us a good set of tools to investigate downstream effects of PKC phosphorylation (Puls *et al.*, 1997; Saleh *et al.*, 2001; Serrano *et al.*, 2008). We hypothesized that in prenatal cocaine exposed brain, elevated phosphorylation of GRIP by prolonged activation of PKC is the result of elevated PDK1 and/or DAG levels due to the down regulation of DGK. We also proposed that this prenatal cocaine effect is reversible; hence inhibition of PKC activity may be effective in restoring AMPAR transmission maturation and attenuating some long-lasting prenatal cocaine-induced neurobehavioral abnormalities.

Since enzymes often localize in the proximity of their substrates, we found that the blockade of GRIP-associated PKCs effectively reduces phosphorylation levels of GRIP and restores GluR2 synaptic surface expression to control level, suggesting that PKC-medicated phosphorylation on the anchoring protein GRIP is a prominent mechanism through which *in utero* cocaine may dramatically shape AMPAR-regulated synaptic transmission. The prolonged activation of GRIP-associated PKC by prenatal cocaine exposure is the result of increased synaptic DAG,

reduced level of synaptic DGK, as well as increased PDK1.

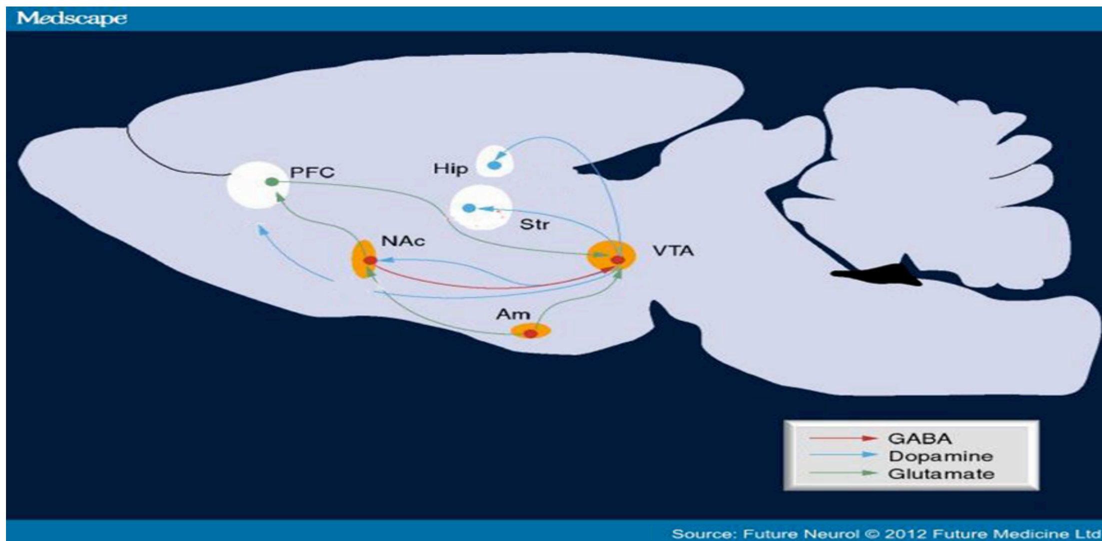

Figure 1. The main circuit for Reward.

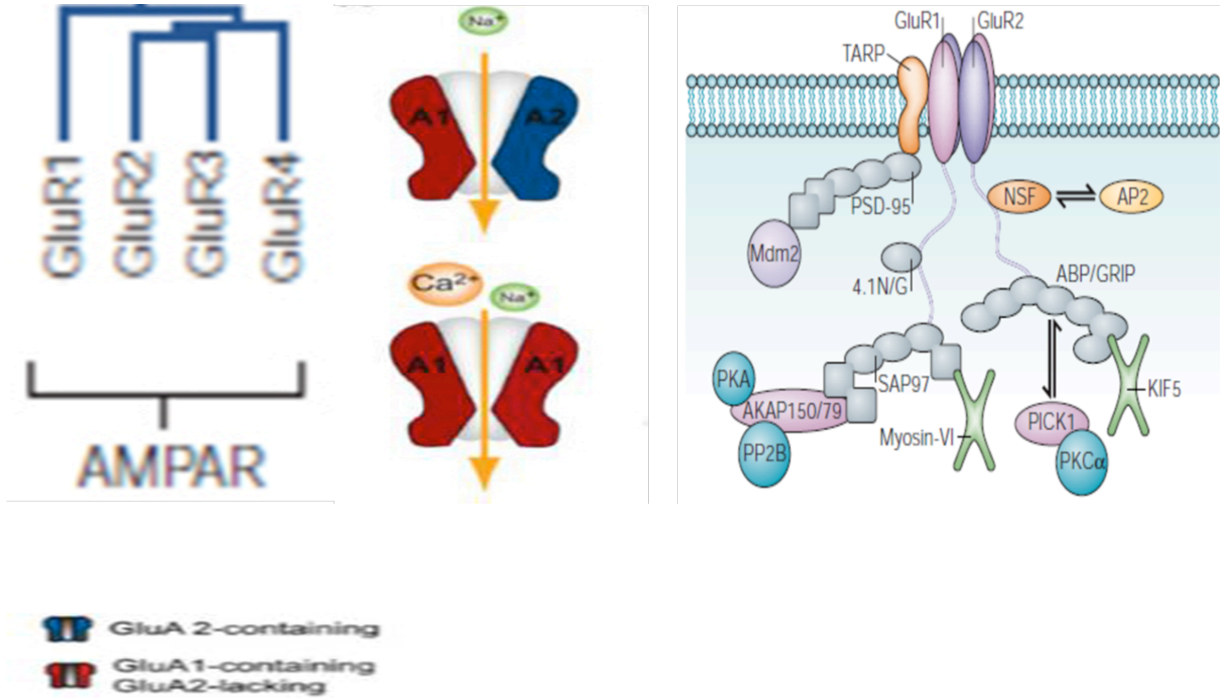

Figure 2. Glutamate receptor structure and its adapting proteins.

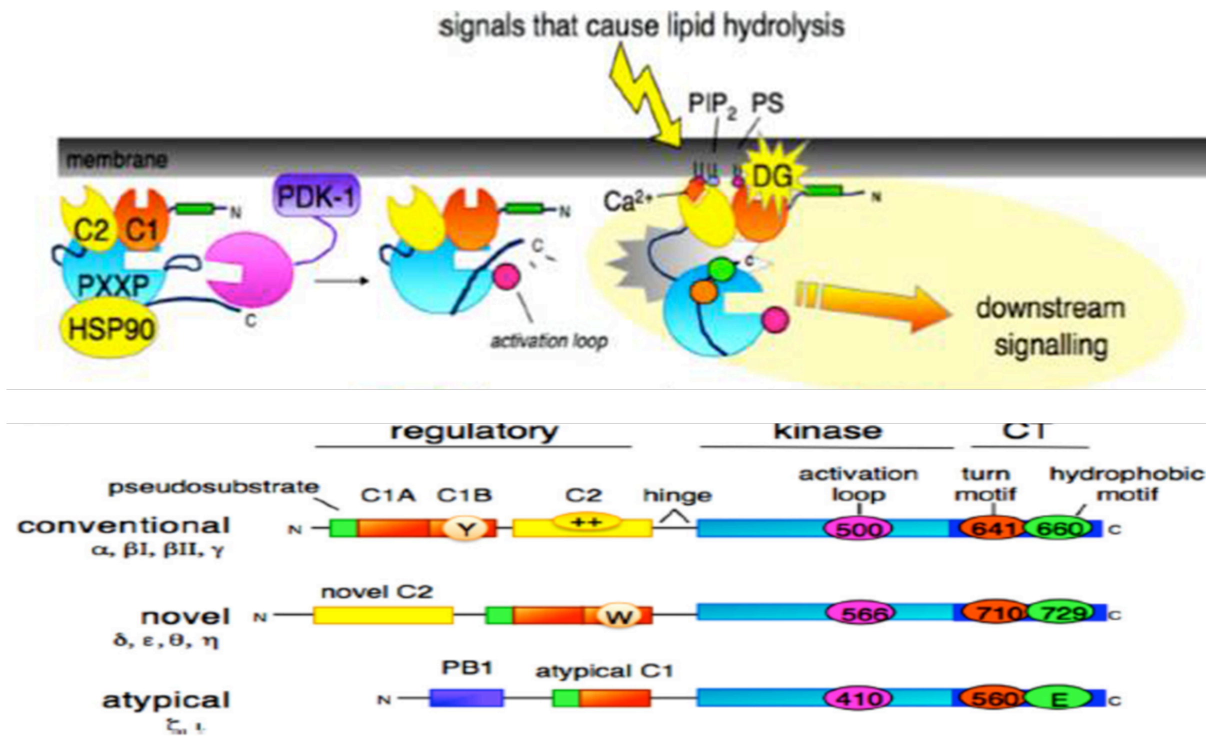

Figure 3. PKC structure and activation by PDK1 and DAG.

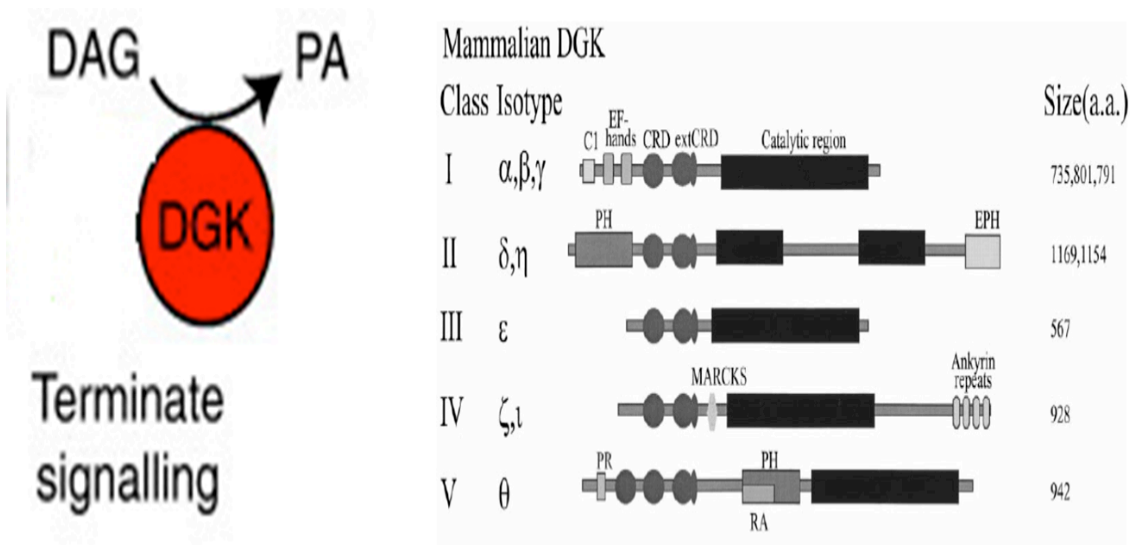

Figure 4. Mammalian diacylglycerol kinases. CRD, cysteine-rich domain; extCRD, extended CRD; PH, pleckstrin homology domain; EPH, EPH C-terminal tail homology domain; MARCKS, sequence homologous to the MARCKS phosphorylation site domain; RA, so-called Ras-associating domain.

Class	Isozym	MW	Tissue expression
I	α	80-86 kDa	Glial cells that form myelin in the white matter (not neuron)
	β	90kDa	Striatum medium spiny neurons; Hippocampal pyramidal cell membrane; Nucleus accumbens; Caudate putmen; Olfactory bulb
	γ	90kDa	Hippocampal pyramidal cell cytosol and dendrites; Cerebellar purkinje cells; Cerebellum
II	δ	130kDa	Not in brain
	η	130kDa	Ubiquitous expression in brain
III	ε	64kDa	Ubiquitous expression in brain
IV	ζ	104kDa	Hippocampus C1 and C3; Cerebral cortices; Olfactory bulb; Cerebellum; Nuclear
	ι	130kDa	Ubiquitous expression in brain
V	θ	110kDa	Ubiquitous expression in brain

Table 1. Tissue distribution of mammalian diacylglycerol kinases.

The distribution of DGKs is depicted using mRNAs in situ hybridization and

immunocytochemistry in rat brain.

DGKα is expressed in glial cells in the white matter, but not neurons.

DGKβ mRNA is observed in the neurons of the caudate-putamen, accumbens nucleus, olfactory bulb, and hippocampus pyramidal cell layer.

DGKγ mRNA is detected most intensively in the cerebellar Purkinje cells, moderately in the septum, hippocampal pyramidal cell layer, and olfactory bulb.

DGKζ subtype is most abundantly expressed in the brain, especially in the cerebellar cortex, hippocampus, cerebral cortex, and olfactory bulb, and weak signals were homogenously observed throughout the brain.

DGKθ and ε show ubiquitous expression in the gray matter.

DGK-β, γ and ζ are abundantly present in brain.

Chapter 2. Experimental Results

Prenatal cocaine exposure increases GRIP-associated activated PKCγ and PKC/PKMζin frontal cortex of postnatal day-21-old (P21) rats

Our earlier work identified that association of the activated PKCγ, PKC/PKMζ and to a less degree PKCa with GRIP1 was increased in prenatal cocaine exposed rat brains (Bakshi *et al.*, 2009). Similarly, here we show that activated PKCγ, PKC/PKMζ and PKCa association with GRIP2 are also increased in frontal cortex of prenatal cocaine exposed brains (figure 5). Concurrently, an increased membrane associated PKC isozymes were noted (Bakshi et al., 2009). In sumary, we show that *in utero* cocaine exposure promotes PKCγ, PKC/PKMζ membrane translocation and activation, and their association with both GRIP1 and GRIP2 highly likely to lead to the protracted hyperphosphorylation of GRIPs. This finding is in consistence with the finding that increased phosphorylation on PKC activation loop by acute cocaine injection (Xue *et al.*, 2012).

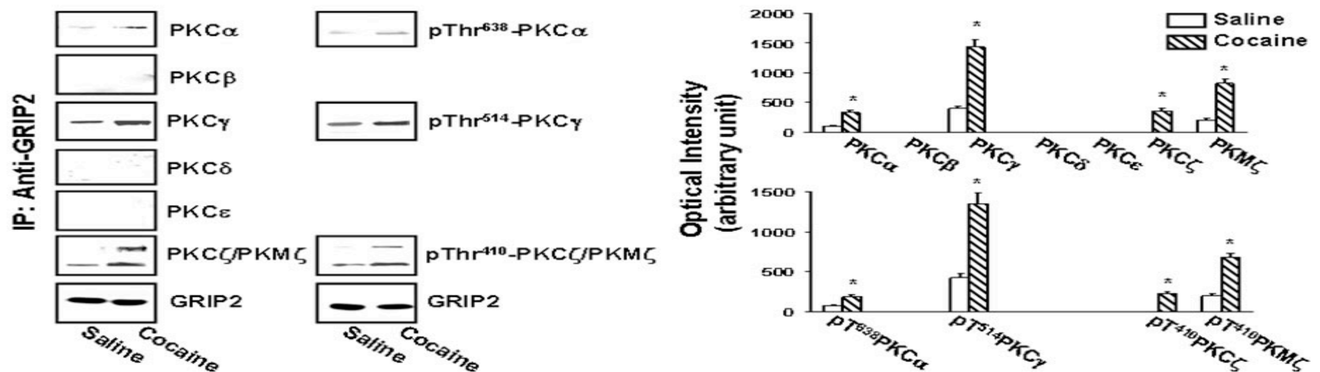

Figure5. Prenatal cocaine exposure markedly increases GRIP2-coupled selective activated PKCα, PKCγ, and PKC/PKMζ.

A. Western blot analysis of the overall and activated PKC/M isozymes associated with GRIP2 in synaptic membranes from prenatal saline- and cocaine- exposed P21 rat brains. B. Densitometric quantification of blots.

PKCγ or PKC/PKMζ inhibition reduces phosphorylated GRIP1 and restores GluR2 synaptic expression

Early work suggested that heightened GRIP phosphorylation mediated by GRIP-associated PKCs leads to decreased synaptic membranous expression of AMPAR subunits GluR2 in frontal cortex from prenatal cocaine-exposed rats (Bakshi *et al.*, 2009). In this research, we blocked GRIP-associated PKCs activity selectively, *in vivo* and *in vitro*, using subtype-specific tissue-permeable PKC pseudosubstrate inhibitors in order to confirm the effects of hyper-activated PKCs over GRIP1 phosphorylation and GluR2 synaptic insertion, and to test the reversibility of those downstream consequences of elevated PKC activity by in utero cocaine exposure.

Firstly, we investigated the duration of inhibition over PKCs by PKC psudosubstrate inhibitors, M-GIP and M-ZIP, and their specificity *in vitro*. Organotypic culture of the frontal cortical slices obtained from P21 prenatal cocaine- or saline-exposed rats were incubated with M-GIP or M-ZIP individually for 1 hour. Myristoylated scrambled peptide (M-SCP) and vehicle were used as the negative control. Subset of slices was removed after treatment each day for analysis. Identical procedure was performed for the next two days. Each day the removed subset of brain slices were fractionated to obtain the synaptosomes. Immunoprecipitates of anti-GRIP1were prepared from the synaptosomes and analyzed by Western blotting with specific antibodies for activated PKCγ/PKCζ/PKMζ (with phosphorylated activation loop) and phosphorylated GRIP on serine/threonine residue. Figure 6 shows that there are higher levels of phosphorylated GRIP1 and of GRIP-associated phosphorylated PKC in frontal cortex of *in utero* cocaine-exposed rats compared to those of saline controls. One hour treatment in day 1 with M-GIP or M-ZIP was sufficient to decrease active PKCγ or PKC/Mζ level respectively and overall phosphorylated

GRIP1 in prenatal cocaine-exposed brain tissue to near control levels. The reduced phosphorylation of PKCs and GRIP1 stayed as low in day2 and day3 and no cumulative inhibitory effect was observed in latter days. The results show a rapid and efficient inhibition of M-GIP or M-ZIP on its respective PKC subtype. The inhibitory potency of either inhibitor lasts for at least 16 hours (the interval between each treatment) although neither pseudosubstrate PKC inhibitor reduced the active PKC level below the control level. These data demonstrate that the increased active PKCγ and PKC/Mζ and their heightened association to GRIP1 are responsible for the persistent GRIP1 hyper-phosphorylation noted in prenatal cocaine exposed rat brain.

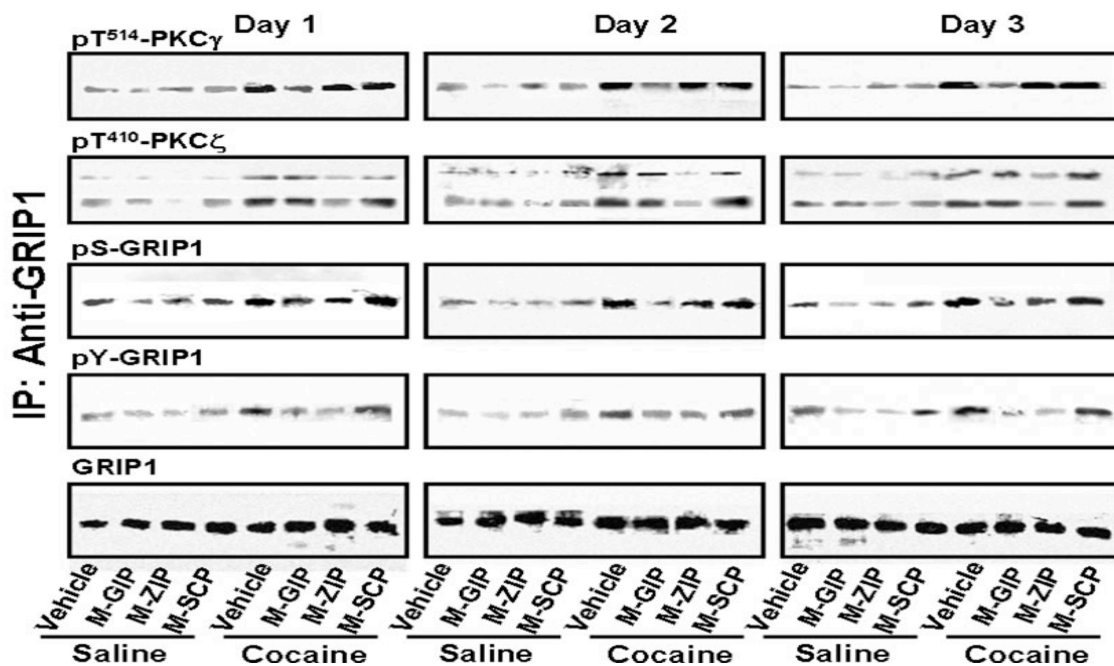

Figure 6. Pseudosubstrate inhibitors targeting PKCγ or PKC/Mζ are equally effective in reducing phosphorylation of GRIP1 by *in vitro* administration in organotypic frontal cortical slice cultures. Frontal cortical slices were prepared from P21 prenatal cocaine- and saline-exposed rats. After 2-day incubation at 37°C in serum-supplemented medium, brain slices were serum-deprived and treated with M-GIP, M-ZIP, or scramble peptide M-SCP for 1 hour. Tissues were then transferred to serum-supplemented medium for 16 hr. Subset of slices was removed each day after treatment, lysed, and anti-GRIP1 immunoprecipitates were prepared and analyzed by western blotting with specific antibodies targeting phosphorylated activation loop of PKCγ/PKC/PKMζ and

phosphorylated GRIP1. Identical procedure was performed for the latter two days.

By administrating PKC psudosubstrate inhibitors *in vivo*, we tested the hypothesis that *in utero* cocaine induced GRIP hyper-phosphorylation is reversible by suppressing the heightened PKC activity in P21 prenatal cocaine-exposed rat brains. In accord, M-GIP and M-ZIP were injected directly into the left frontal cortex and scrambled peptide M-SCP was administered into the contralateral frontal cortex as control. Similar to the results obtained using organotypic slice culture, administration of M-GIP and M-ZIP robustly and specifically attenuated PKCg and PKC/Mz activities, respectively (figure 7). Moreover, both inhibitors are equally effective in attenuating the levels of serine- and tyrosine- phosphorylated GRIP1 in the prenatal cocaine exposed brains. Together with the data summarized in figure 6, these data strongly support the notion that hyper-activated PKCg and PKC/Mz are prominent mediators of the persistent GRIP phosphorylation in the prenatal cocaine-exposed brains.

Figure 7. Pseudosubstrate inhibitors targeting PKCγ or PKC/Mζ are equally effective in reducing phosphorylation of GRIP1 by being administered intra-cortically. M-GIP or M-ZIP was directly injected into

one-side of frontal cortices of P21 prenatal saline- and cocaine-exposed rats. The contralateral side of frontal cortex received either vehicle or scrambled peptide (M-SCP) to serve as controls. Rats were sacrificed 16 hours after the injection. The levels of activated PKCγ, PKC/Mζ, as well as phosphorylated GRIP1 were analyzed in anti-GRIP1 immunoprecipitates from the frontal cortical lysates by western blotting**.**

Thirdly, to confirm that hyper-activation of GRIP-associated PKC is responsible for the decreased GluR2 synaptic insertion (Bakshi *et al.*, 2009; Bakshi *et al.*, 2011), we assessed the cellular distribution of GluR2 in prenatal cocaine exposed rats following administration of pseudosubstrate PKC inhibitors into frontal cortices. Synaptosomes derived from frontal cortices were separated into synaptic membranous and cytosolic fractions so as to measure the levels of GluR2 in each fraction. Separately, membranous and cytosolic fractions were also prepated from synaptosomes derived from organotypic frontal cortical slice cultures incubated with M-GIP, M-ZIP, or scrambled control peptide. In frontal cortices of prenatal cocaine-exposed rats, membrane-localized GluR2 was lower with corresponding higher cytosolic GluR2 compared to that in saline-exposed controls (Figure 8 and 9). Administration of M-GIP or M-ZIP, both *in vivo* (frontal cortical injection) and *in vitro* (organotypic slice cultures)*,* effectively restored the level of membrane-associated GluR2 in prenatal cocaine-exposed rat brains to near control levels without alterations in overall GluR2 expression (Figure 8 and 9). The restoring of synaptic GluR2 by PKC inhibition reinforces the conclusion that hyper-activated PKC with corresponding increased GRIP1 phosphorylation is the primary underlying mechanism responsible for the altered AMPAR transmission observed previously (Bakshi et al., 2009; Figure 9). These data resonate with the data summarized in figure 1 to 5 that directly link hyper-activation of PKCγ/PKCζ/PKMζ to GRIP hyper-phosphorylation and ultimately reduction of GluR2 synaptic expression in prenatal cocaine-exposed brains. We propose that PKC hyper-activation as the underlying mechanism responsible for AMPAR dysfunction and perhaps consequent biological

behavioral abnormalities in prenatal cocaine-exposed animals.

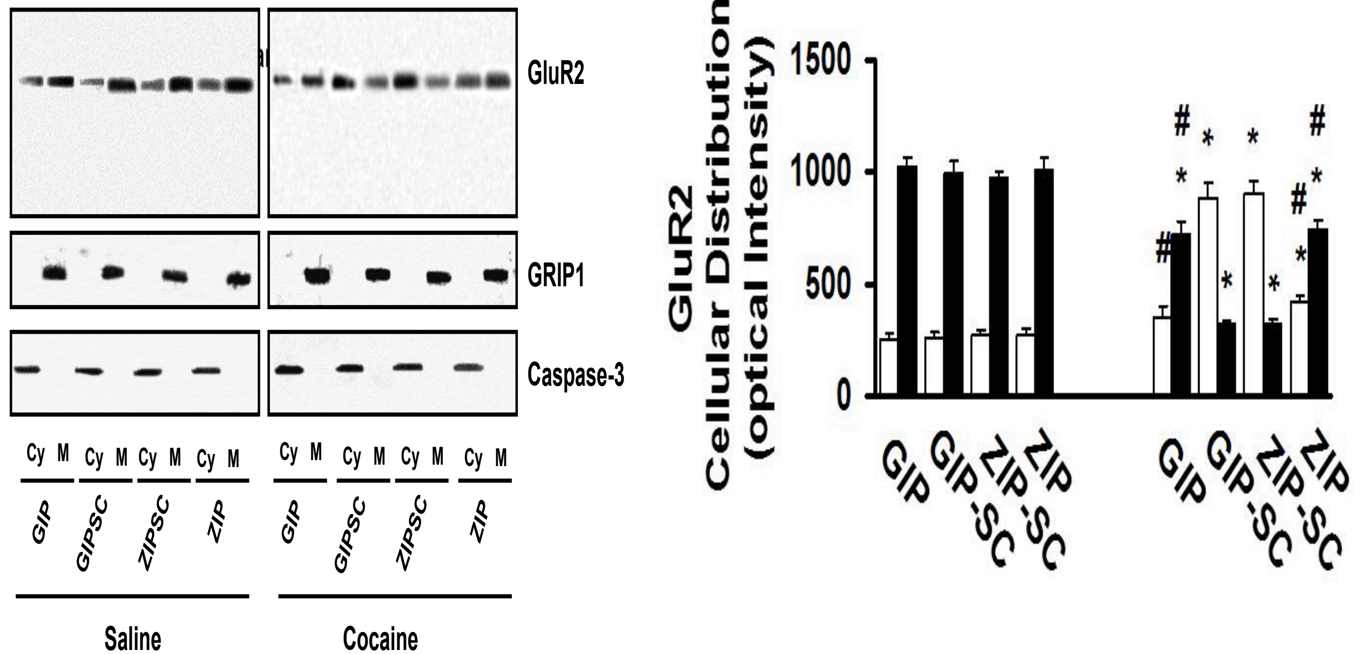

Figure 8. *In vivo* Pseudosubstrate inhibition of PKCγ or PKC/Mζ are equally effective in restoring GluR2 on the membrane.. *p< 0.01, **p< 0.05 compared to respective value in saline group. #p<0.01 compared to respective value in SC treated cocaine group**.**

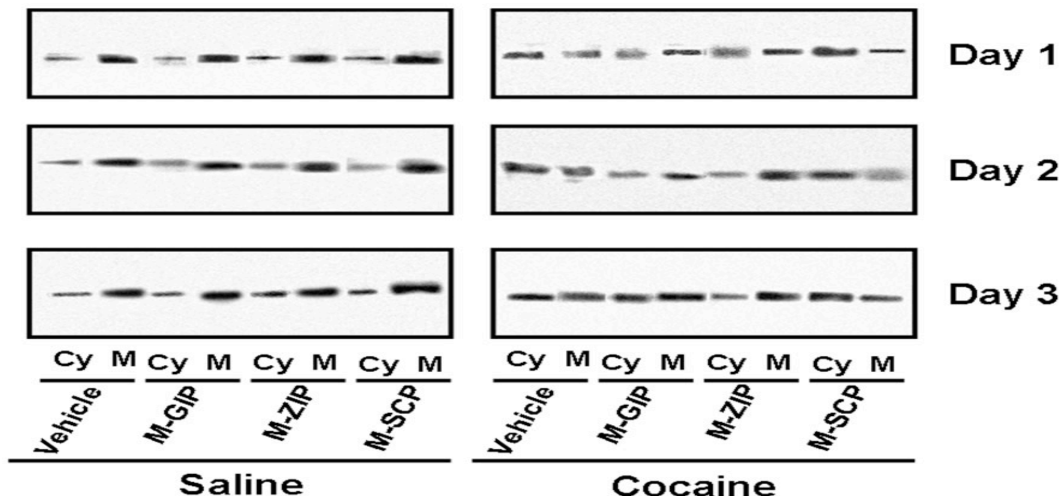

Figure 9. Pseudosubstrate inhibitors targeting PKCγ and PKC/Mζ, but not the scrambled peptide, restore GluR2 surface expression in prenatal cocaine-exposed brains. Organotypic frontal cortical slice cultures were

prepared as described in figure 2. Cytosolic and membranous fractions of the synaptosomes were prepared and western blotting was performed with antibody against GluR2.

Prenatal cocaine exposure elevates DAG concentration in synaptic membrane fractions of frontal cortex compared to prenatal saline exposed/naive control rats

Our previous data show elevated PKC activity promoted by *in utero* cocaine exposure. All PKC isozymes' translocation and duration of being tethered on the membrane is positively correlated with DAG in the membrane (Asaoka *et al.*, 1992; Huang & Huang, 1993; Ron & Kazanietz, 1999). Therefore, we expected a higher level of DAG in frontal cortical membranes of in utero cocaine-exposed rats comparing to control animals. DAG was extracted from either the entire frontal cortical tissue or from synaptic membrane fractions. The extracted DAG was converting into phosphatidic acid (PA) in the presence of exogenous DAG kinase and radioactive ATP. [γ-^{32}P] phosphatidic acid (PA) converted from DAG was quantified against a linear standard curve built by using purified exogenous DAG as substrates (giving the equation x (μmol/mg) =y/6654.3/398.58). A new extraction method with pure hexane was developed and the extracted DAG was dried and re-suspended in reaction buffer containing 5 mM α-cyclodextrin. The results show that *in utero* cocaine exposure increased DAG concentration in the frontal cortex by 38.65% (Figure 10). There was 8.43±0.82 nmol DAG extracted from per 1 mg protein from frontal cortex of prenatal cocaine exposed animals while 14.88±2.23nmol/mg from saline control and 2.12 nmol/mg from naïve rats. Further we fractionated frontal cortex tissue to obtain the membranous fraction and measured the DAG concentration per 1 mg membranous protein. The data show that in utero cocaine increased DAG concentration in synaptic membrane of P21 rats' frontal cortex by 75.19% (Figure 11). Prenatal cocaine exposure dramatically increased DAG mass in rat's frontal cortex particularly in the synaptic membranous compartments. It explains the observed elevated membranous GRIP-associated PKCs and their hyper-activation.

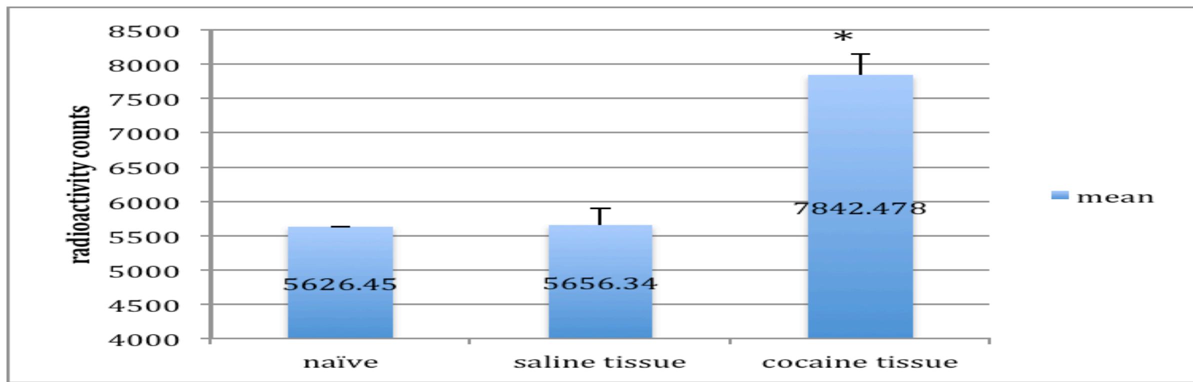

Figure 10. Prenatal cocaine-exposure produces elevated DAG mass in rat's frontal cortical compared to saline control. DAG was extracted from prefrontal cortical tissues from P21 naïve and prenatal saline- and cocaine-treated rats. Extracted DAG was phosphorylated by exogenous DGK with the presence of [γ-^{32}P] ATP. PA derived from DAG was separated by TLC and the radioactivity was assessed by liquid scintillation counter. Our results show an increased DAG level in the frontal cortex tissue of P21 prenatal cocaine-treated rats. n=6. Data are means ± s.e.m. of the radioactivity counts per 1mg protein from frontal cortical tissues. *p= 0.0052, compared to respective protein in the saline-treated group.

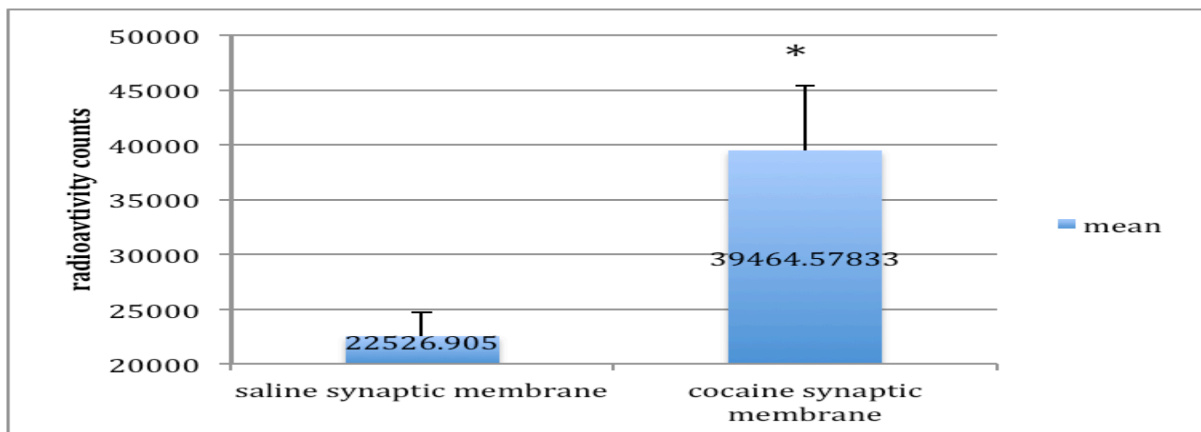

Figure 11. Prenatal cocaine-exposure produces elevated DAG mass in rats' frontal cortical synaptic membranes compared to saline control. Frontal cortex tissue from P21 prenatal saline- and cocaine- treated rats was fractionated to yield the membranous fraction. DAG was extracted from the membranous fractions and measured the same way as figure 5. Our results show an increased DAG level in the frontal cortex synaptic membrane of P21 prenatal cocaine-treated rats. n=6. Data are means ± s.e.m. of the radioactivity counts per 1mg protein from frontal cortical membranes. *p= 0.02285, compared to respective protein in the saline-treated group.

Prenatal cocaine exposure reduces GRIP-associated DGKγ and DGKζ levels

DGK is the primary regulator of membrane DAG mass. DGK phosphorylates DAG into PA and in turn PKC is released from membrane because of the interrupted tethering onto DAG. Therefore DGK serves as an indirect mediator regulating PKC activity. An elevation in DAG amount and PKC activity suggest defective DGK in prenatal cocaine-exposed brains. DGK activation, as for PKC, requires trafficking of the enzyme from cytosol to membrane where it associates with scaffolding proteins (van Baal *et al.*, 2005). Here we identified DGK isozymes that are associated with GRIP in order to investigate whether DGK plays a role in the regulation of GRIP-associated PKC within the GRIP1/2- PKC signaling complex and if yes how it is affected by prenatal cocaine exposure. We find that isozymes DGKγ and DGKζ are associated with GRIP1 and GRIP2 (Figure 12). This association of DGK to GRIP is markedly reduced by prenatal cocaine exposure. Since DAG level in membranes is a primary determinant of the duration for PKC in the membranes, our data suggest that a reduced DGKγ/DGKζ-GRIP association in prenatal cocaine-exposed brain may be responsible for the sustained DAG as well as GRIP-associated PKC membrane localization.

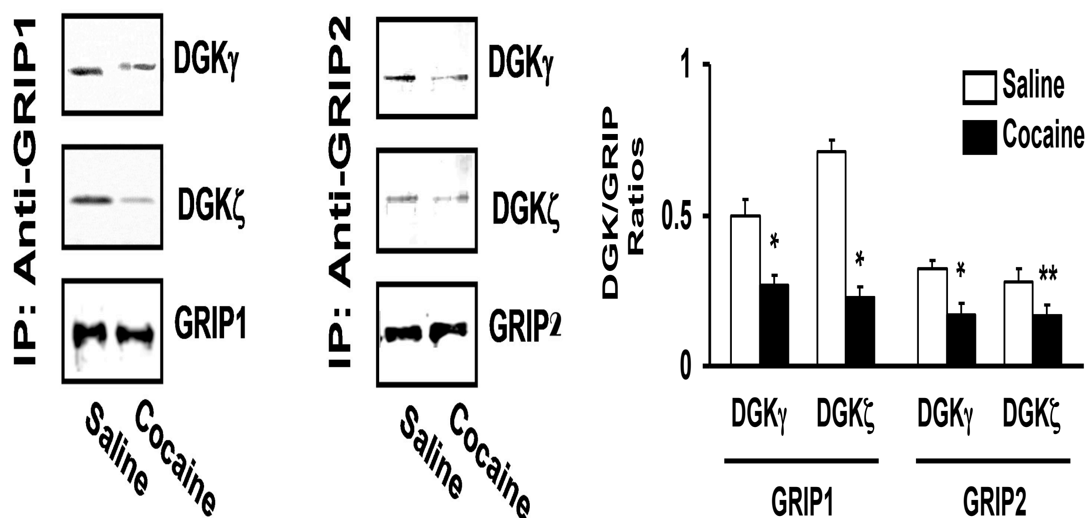

Figure 12. Prenatal cocaine exposure reduces the association of GRIP with DGKγ and DGKζ in frontal cortex

of P21 prenatal cocaine-exposed rats.

Besides the association of DGK isozymes with GRIP, their expression and distribution in the synapses and their kinase activity were examined. Frontal cortical synaptosomes were fractionated into membranous and cytosolic compartments. Western blotting illustrated in figure 13 shows that in prenatal cocaine-exposed brains, both cytosolic and membranous expression levels of DGKγ and DGKζ were decreased by approximately 50% without discernible changes in GRIP1 and caspase-3 expression. The reduced GRIP-associated DGKγ and DGKζ in prenatal cocaine-exposed brains could be the result of reduced synaptic expression.

In addition to changes in synaptic expression and GRIP-association of DGK, prenatal cocaine exposure may also alter DGK catalytic activity. Kinase activity of synaptic DGKγ or DGKζ from frontal cortical tissues was therefore measured using an *in vitro* kinase assay with the presence of exogenous substrate DAG and radioactive ATP (Bunting *et al.*, 1996). DGK was purified by immunoprecipitaion from synaptosome lysate. Data in figure 14 show that the kinase activity of DGKζ decreases approximately 50.8% in prenatal cocaine exposed rats compared to saline control (cocaine 8.66×10-3 nmol/min/mg versus saline 17.59×10-3 nmol/min/mg) while there is no significant change in kinase activity of DGKγ (cocaine 6.39×10-3 nmol/min/mg versus saline 6.21×10-3 nmol/min/mg). Combined with the decreased expression levels of DGK (Figure 13), the overall output of GRIP- associated DGK's catalytic ability to phosphorylate DAG decreased intensively in prenatally cocaine-exposed rat frontal cortex compared to control animals, which accounts for the observed elevated DAG mass and GRIP- associated PKC membrane-translocation at synapses.

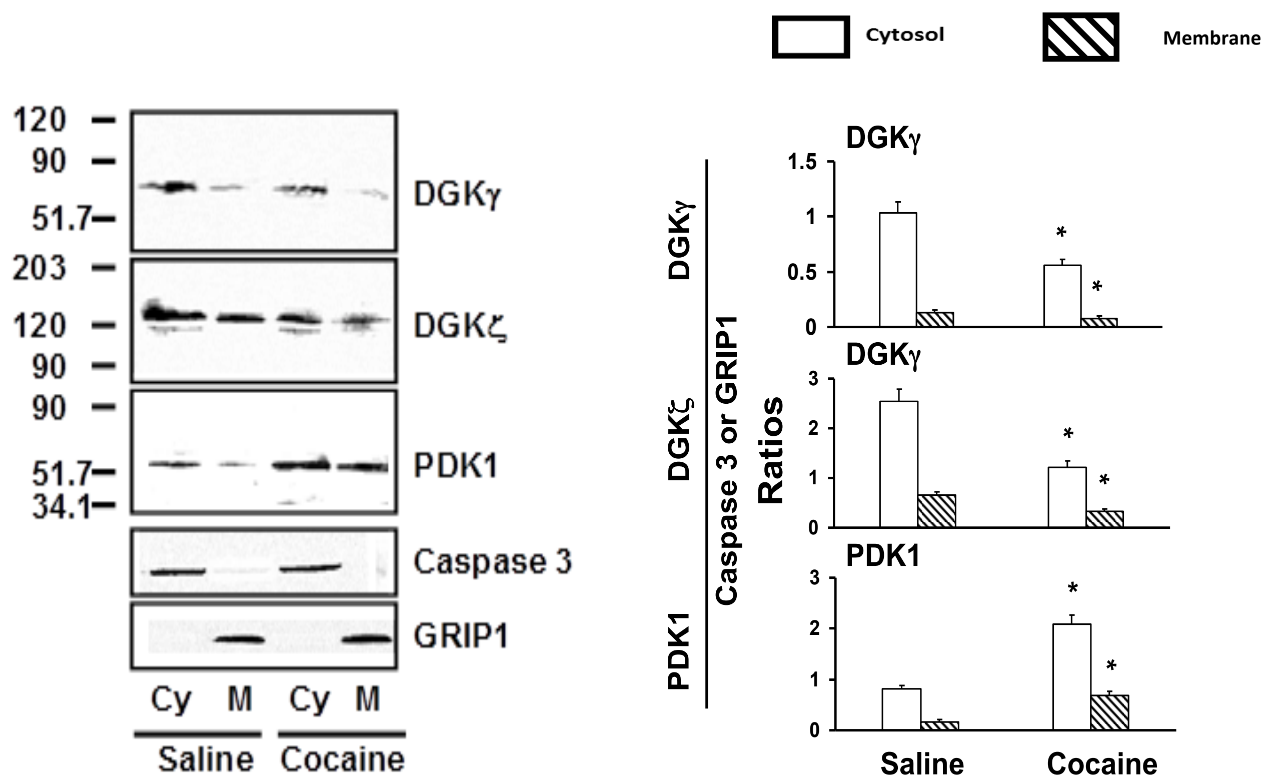

Figure 13. Cellular distribution of DGKγ and DGKζ isozymes in frontal cortex from P21 prenatal saline- and cocaine- exposed rats. DGK show 50% decrease and PDK1 peaked up 3 folds. The expressions of DGK and PDK1 in cytosolic and membranous fractions of frontal cortical synaptosomes were measured by western blotting with specific antibodies targeting specific DGK isozymes and PDK1. The blots were stripped and reprobed with anti-GRIP or -Caspase 3 as control of loading of membrane and cytosolic fractions.

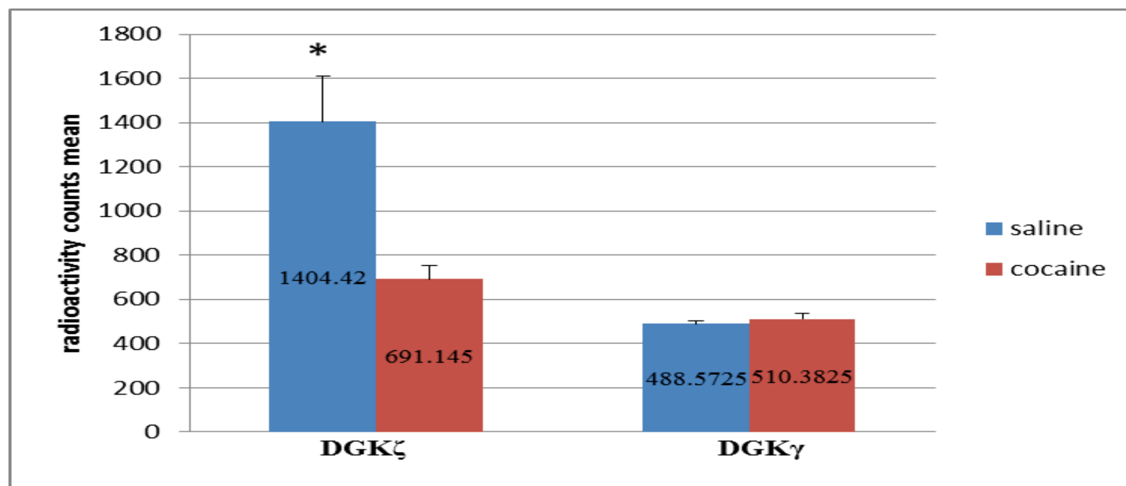

Figure 14. Prenatal cocaine exposure reduces kinase activity of DGKζ without altering DGKγ activity in

frontal cortex of P21 rats. DGK isozymes were first purified by immunoprecipitation and their kinase activities were determined by measuring radioactive phosphate incorporation into PA with presence of [r-^{32}P] ATP and exogenous standard DAG as substrates. Synaptosome fractions were prepared first and solubilized by triple detergent: 0.5% NP-40, 0.2% sodium cholate, 0.5% digitonin. Protein concentrations of lysates were checked by Bio-Red. The same amount of protein (1mg) was taken and the volume of each sample was made up with IP buffer for immunoprecipitation. Obtained immunoprecipitates were re-suspended for kinase reaction in the same reaction buffer as for the measurement of DAG. Our results show a decreased DGKζ activity without altering DGKγ in the frontal cortex tissue of P21 prenatal cocaine-treated rats. n=4. Data are means ± s.e.m. of the radioactivity counts per 1mg protein from frontal cortical synaptosome. **p*= 0.01597, compared to respective protein in the saline-treated group.

Prenatal cocaine exposure increases PDK1 in frontal cortex

Because of the observed higher level of phosphorylation on the activation loop of GRIP-associated PKC (pT^{514}-PKCγ, pT^{410}-PKC/Mζ and pT^{638}-PKCα) in frontal cortex of prenatal cocaine-exposed rats, the upstream kinase PDK1, who catalyzes this phosphorylation, was expected under an up regulation by *in utero* cocaine. The cellular distribution of PDK1 shows a markedly higher level of PDK1 of about 3 folds increase in both cytosolic and membrane fractions of frontal cortical synaptosomes of P21 prenatal cocaine-exposed rats (figure 13). The changes in PDK1 kinase activity were addressed by a biochemistry assay. PDK1 was purified by immuneprecipitation from solubilized frontal cortices. Exogenous de-phosphorylated SGK was added as the substrate for phosphorylation in the presence of [g-^{32}P]ATP. The levels of PDK1 and phosphorylated SGK (p-SGK) were simultaneously determined by Western blotting with anti- PDK1 plus anti- p-SGK. The ration of p-SGK/PDK1 was then quantified. The data obtained indicate that the activity of PDK1 derived from frontal cortices of P21 prenatal cocaine-exposed rats was decreased by 51.5% when compared to that of the saline-treated group (Figure 15).

Naïve tissue and 100 ng exogenous PDK1 was similarly immuneprecipitated and assayed to serve as the references. The data obtained suggest that a reduced PDK1 activity may be the trigger of the compensatory increase in PDK1 expression in synapses of prenatal cocaine-exposed brains. The increased PDK1 expression appears to more than compensated for the reduced kinase activity since PDK1 mediated PKC activation indicated by the increased phosphorylation in PKC activation loop is increased by prenatal cocaine exposure ((Bakshi *et al.*, 2009). Together with the elevated DAG level in synaptic membranes, these results suggest that prenatal cocaine exposure promote PKC activation via multiple mechanisms. These include (1) sustained membrane localization and association with of GRIP- associated PKCs due to an increased DAG in membranes caused in part by the reduced GRIP-associated DGKγ and DGKζ and (2) increased membrane-tethered PDK1 level.

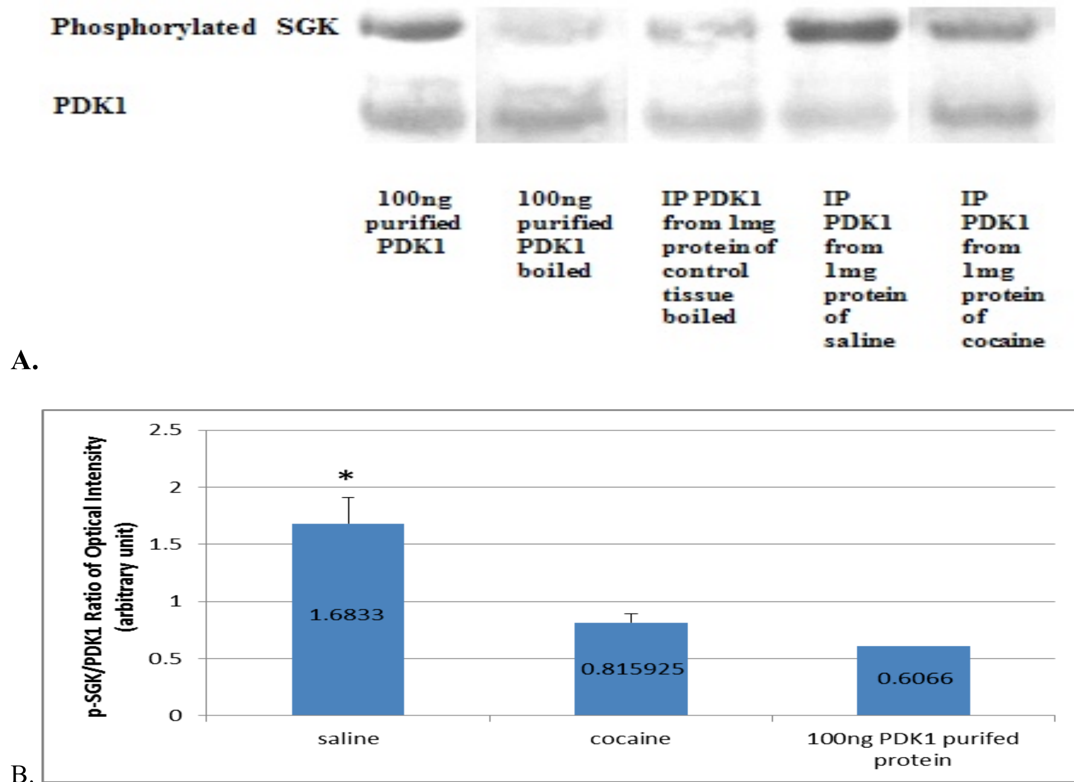

Fig 15. Prenatal cocaine exposure reduces synaptic PDK1's kinase activity 50%. (**A**) The effect of prenatal cocaine exposure on PDK1 kinase activity was evaluated by the phosphorylation level of exogenous SGK as

substrate. The levels of purified PDK1 and p-SGK were determined by Western blotting. Our results show that PDK1 purified from frontal cortical synaptosome has a reduced kinase activity in P21 cocaine-treated rats compared to saline control. (**B**) Densitometric quantification of blots. The data are expressed as the ratios of p-SGK optical intensity normalized by the optical intensity of purified PDK1. n = 8. Data are means ± s.e.m. of the ratios of p-SGK to PDK1 optical intensities. *p=0.00548, compared to respective enzyme purified from the saline-treated group.

Chapter 3. DISCUSSION

Exposure to cocaine during gestation was shown to disrupt AMPAR transmission in frontal cortex of P21 rats (Bakshi et al., 2009). In the brains of the prenatal cocaine-exposed P21 rats, a dramatic reduction in synaptic expression of GluR2 was shown (Bakshi et al., 2009). GluR2 containing AMPAR are normally stabilized at the synapses by binding to scaffolding protein GRIP1/2 while cycling on and off the synaptic membranes constitutively. Disruption of the association between GluR2 and GRIPs as noted in prenatal cocaine-exposed brains results in loss of synaptic AMPARs. Among various possible mechanisms, PKC-mediated phosphorylation on S^{880} of GluR2 was suggested to be a prominent mechanism to disrupt the association between GluR2 and GRIP (Matsuda *et al.*, 2000). However, our earlier data derived from prenatal cocaine-exposed brains indicate that in prenatal cocaine exposed P21 rat brains, the reduced synaptic membrane-associated GluR2 is the result of an increased PKC-mediated GRIP phosphorylation of the GRIPs rather than GluR2s since pS^{880}GluR2 level was not altered in prenatal cocaine-exposed brains (Bakshi *et al.*, 2009).

To further elucidate the underlying mechanism responsible for hyper-activated PKCs that apparently leads to hyper-phosphorylation of GRIPs, reduced GluR2 synaptic expression, and defects in AMPAR transmission, we employed subtype-specific pseudopeptide PKC inhibitors in this study. We further proposed that this *in utero* cocaine exposure induced alteration in AMPAR synaptic transmission may be normalized by removal of excessive PKC activities. To this end, we found that administration of PKC pseudo-substrate inhibitor peptides GIP or ZIP, either *in vivo* or *in vitro*, effectively reduce phosphorylated GRIP1 levels and restore membrane-associated GluR2 (Figure 2, 3, 4 and 5). These data together provide a straight forward

explanation that hyper-activated PKCs are the primary contributor of the sustained GRIP phosphorylation and the consequent AMPAR synaptic dysfunction in the prenatal cocaine-exposed brains. Consistent with our findings, in transfected cells, GluR2 surface insertion was found to be interrupted by GRIP phosphorylation on serine 917 mediated by PKC (Kulangara *et al.*, 2007). The close association between PKCγ and PKC/PKMζ activation and GRIP phosphorylation and the fact that they are co-localized with each other suggest that elucidate the underlying mechanism responsible for hyper-activated PKC may provide a valuable avenue to enhance our understanding of the neurobehavioral abnormalities in prenatal cocaine-exposed subjects.

The de-phosphorylation of GRIP following incubation with GIP or ZIP occurs rapidly by day1, suggesting a tight regulation of the GRIP phosphorylation level by the phosphatases (Figure 2 and 3). The reduced GRIP phosphorylation leads to improved GluR2 level in the synaptic membranes and consequent reduction of cytosolic GluR2 (Figure 4 and 5). These data together reinforce the notion that PKC-mediated GRIP phosphorylation is a critical regulator of the GluR2 synaptic targeting, and its derangement underlies AMPAR synaptic transmission defects in the prenatal cocaine-exposed brains. Importantly, pseudosubstrate PKC inhibitors did not affect GluR2 synaptic expression in prenatal saline-exposed rats (Figure 4 and 5) despite a mild inhibition on activated PKC and slightly lower phosphorylated GRIP1 level were observed (Figure 2 and 3). These data may imply that PKC inhibitors or any other methods leading to PKC inhibition could have therapeutic value for adolescents who have had exposed to cocaine *in utero*.

PKCγ, PKC/PKMζ, and GluR2 were identified associate with GRIP1 in frontal cortex of P21 rats. Both GRIP1 and GRIP2 are substrates for PKC phosphorylation (Bakshi *et al.*, 2009).

Consistently with earlier reports that GluR2s are associated with GRIP1 in transfected cells (Dong *et al.*, 1997) and in rat brain (Wyszynski *et al.*, 1999), our results show not only GluR2s are linked to GRIPs but also PKCγ and PKC/PKMζ as well as DGKγ and DGKζ are associated with GRIP2 as well as GRIP1 (Figure 1-PKC, Figure 8-DGK). Despite the fact that GRIP1 and GRIP2 are the products of different genes, GRIP2 is highly homologous with GRIP1 and can form hetero-multimers with GRIP1 to allow the formation of very large macromolecular complexes (Dong *et al.*, 1999). Either GRIP1 or GRIP2 can sustain LTD expression in cerebellar Purkinje neurons (Takamiya *et al.*, 2008). Therefore GRIP1/2 probably participate forming AMPAR-GRIP-PKC-DGK signaling complex, where regulation of signaling events can be precisely controlled, although GRIP2 was not coimmuneprecipitated with GluR2 as GRIP1 was (Dong *et al.*, 1999).

AMPARs are tetramers made of the subunits GluR1 to GluR4 (Hollmann & Heinemann, 1994). GluR2 is the subunit that governs permeability to calcium of AMPAR (Hollmann *et al.*, 1991; Burnashev *et al.*, 1992; Wolf & Tseng, 2012). The alteration in the synaptic GluR2-containing AMPARs levels regulated by associating with GRIPs in animals and humans that exposed to cocaine prenatally can conceivably influence the electrophysiological property of synapses. In accord with our findings that prenatal cocaine exposure reduces GluR2 synaptic expression, a delay in the switching of AMPA subunit GluR2-lacking receptors into GluR2-containing receptors was noted in the ventral tegmental area (VTA) of prenatal cocaine exposed animals (Bellone *et al.*, 2011). Moreover, GluR2, specifically, insertion into the synaptic membrane promotes spine growth, increase spine size, and enhance synaptic strength during early development (Engert & Bonhoeffer, 1999; Matsuzaki *et al.*, 2001; Kumar *et al.*, 2002; Passafaro *et al.*, 2003). Indeed it was found that either cocaine- or prenatal cocaine-exposed animals have

prominent morphological changes in dendritic spines in the brain (Morrow *et al.*, 2007; Frankfurt *et al.*, 2009; Salas-Ramirez *et al.*, 2010; Frankfurt *et al.*, 2011). Altogether, these findings suggest that GluR2 insertion into the synaptic membranes is a critical regulator of synaptic plasticity that governing proper brain functions and is crucial to the maturation of glutamatergic transmission in brain reward system.

To elucidate the molecular mechanisms responsible for hyper-activated PKCs in the prenatal cocaine exposed brains, we examined PKC's major regulators such as DAG level in synaptic membranes, DGKs that regulate DAG levels, and PDK1 that phosphorylates PKCs on their activation loop and thereby activate them. We found that prenatal cocaine exposure generates a significantly higher DAG concentration in synaptic membranous compartment in frontal cortices with a 75% increase compared to saline-exposed control animals. In the lysate of whole frontal cortical tissues, a 38% increase in prenatal cocaine-exposed animals comparing with saline/naïve control. This sustained increase of synaptic DAG effectively explains the observed heightened synaptic membranous localization of PKC in prenatal cocaine-exposed animals. The heighted increase in synapses suggests that the accumulation of DAG in PKC compartment is precisely regulated. The data also show that DAG's regulation on downstream pathways is achieved through spatial restriction and occurs locally. Consistent with this view, several DAG pools have been described in distinct forms, spatially separated with different efficiency to activate PKC within the cell (Cabot & Jaken, 1984; Ford *et al.*, 1989; Wakelam, 1998; D'Santos *et al.*, 1999). During analysis of DAG concentration, dried DAG extract was solubilized in reaction buffer containing 5mM alfa-cyclodextrin. Comparing to octyl-β-D-glucoside/cardiolipin micelle solution(Preiss *et al.*, 1986), 0.3% Triton X-100detergent solution (Paterson *et al.*, 1991), or sodium deoxycholate (Kato & Takenawa, 1990), we found alfa-cyclodetrin, as a novel detergent

used in lipid extraction which generated the clearest solution of DAG. It is superior to the visible insoluble suspensions while using other detergents. The reason might be that the first two groups extracted DAG from cultured cells, and Kato used exogenous DAG as substrate, while we extracted DAG from rat cortical tissue. PA and/or PS were tested as activators for kinase reaction. However, we did not see significant facilitation. Ca^{2+} was reported as a good activator of DGK at 1mM concentration; however, we found that at 0.5mM concentration Ca^{2+} already exhibits an inhibitory effect over DAG activity. The elevated synaptic concentration of DAG allows prolonged PKC localization on the membrane. However, PKC activation particularly the atypical PKC needs an upstream enzyme PDK1. *In utero* cocaine exposure dramatically increases PDK1 expression in neurosynaptosomes (Figure 9) that is accompanied by a reduced PDK1 activity by 50% in prenatal cocaine-exposed animals (Figure 11). The 3-fold increase in PDK1 expression and the observed increase in PKC/PKMζ phosphorylation in their activation loop suggest strongly that the increased synaptic DAG and PDK1 are responsible for the heightened PKC activation in the prenatal cocaine-exposed brains. DGK is a key determinant of DAG. Normally DAG signaling at synapses is very transient. Increased DAG level is wiped out by DGK rapidly to terminate DAG downstream signaling. The observation with prolonged half-life of DAG at synaptic membranes in non-stimulated tissue of prenatal cocaine-exposed animals suggests a dysregulated DGK. Given that DAG level was found dramatically increased and spatially restricted; we studied GRIP1/2-associated DGK isozyms. Our data show that prenatal cocaine exposure reduces DGKγ and DGKζ expression by approximately 50-60% by prenatal cocaine exposure (Figure 9). In addition, a 50% less DGKγ and DGKζ were found to co-immuneprecipitated with GRIP (Figure 8). This observed reduced GRIP-associated DGK level could be a result of reduced DGK expression as well as a reduced binding affinity with

scaffolding proteins. DGKζ was found phosphorylated by PKC on the MARCKS motif (close to the catalytic domain) that conceivably can produce conformational change of DGK and affect its kinase activity (Bubb *et al.*, 1999; Luo *et al.*, 2003). This may be the cause of reduced DGKζ kinase activity in prenatal cocaine-exposed rats (by approximately 50% compared to the saline controls) whereas there was no significant change in DGK γ (Figure 10). Altogether, prenatal cocaine exposure significantly affects GRIP-associated DGKs in frontal cortex, which explains the elevated synaptic DAG, the sustained membrane- and GRIP-associated PKCs and consequentially hyper-activated GRIP-associated PKCs in this signaling complex.

PKC hyper-activation following upon agonist stimulation is often compensated within a brief span of time by down-regulation from DGK (Shirai *et al.*, 2000). However, this compensatory effect was impaired in prenatal cocaine exposed animals as shown by our data. Increasing evidences show close spatial and biochemical interactions between PKC isosymes and specific DGK subtypes. DGKζ was reported phosphorylated by PKCα on the MARCKS domain resulting in a reduced kinase activity of DGKζ (Luo *et al.*, 2003). DGKθ was reported phosphorylated by PKCε and η (van Baal *et al.*, 2005). Besides the spatial interaction and the implied down-regulation of GRIP-associated DGK by GRIP-associated PKC, the time frame of translocations of PKC and DGK from cytosol to membrane also showed strategically correlated. DAG promotes translocation of both PKC and DGK and tethers them on the membrane. It was long established that PKC and DGK isotypes contain cysteine-rich domains (CRD1 and/or CRD2) through which they localize to DAG (Hurley *et al.*, 1997; Newton, 1997; Houssa & van Blitterswijk, 1998). Evidence shows that DAG promotes DGKθ translocation in a PKC-independent fashion requiring intact CRD domain while activation of PKCε also promotes DGKθ translocation to membrane (van Baal *et al.*, 2005). Like PKC, DGKs also translocate to

membrane in response to rising DAG levels. With application of membrane-permeable DAG analog, PKCγ showed an early translocation from cytosol to membrane following by DGK γ membranous translocation (Shirai *et al.*, 2000). Therefore, it is likely that when the level of DAG rises, PKC is activated by this second messenger and translocates to the membrane at a faster rate than DGK does. The reason for a faster translocation is likely to be CRD domains of PKC has a high affinity for binding to DAG, whereas the CRD domains of DGK is thought to belong to the low affinity group (Shirai *et al.*, 2000; van Baal *et al.*, 2005). The long-lasting hyper-activation of GRIP-associated PKCs in synaptic membranes may therefore in part mediatedmediate by a reduced negative regulation from GRIP-associated DGK. The phosphorylation of DGK by PKC may reduce a catalytic activity change of DGK together with reduced DGK expression or dissociation of translocated DGK from the signaling complex, may attenuate the negative regulation on PKC from DGK. Altogether, we suggest that in the basal state, when DAG levels in the cell are low, certain amount of DGK physically associates with endogenous phosphatidylinositol-specific phospholipase C (PLC) (van der Bend *et al.*, 1994; van Baal *et al.*, 2005) in signaling complexes metabolizing local DAG and prevent PKC activation. Upon stimulation, when PKC activity is required, local DAG levels increase fast transiently to overcome the ability of DGK to remove DAG leading to association of PKCs with the membranes that followed by the DGKs that have CDRs with low affinity for DAG to terminate PKC activation.   This regulation mechanism might serve the purpose of activating either transient or prolonged PKC activation, depending on the given stimulation/information the animal receives, in order to serve different aims such as signal transduction cascades, regulating cell growth and differentiation, apoptosis, and cytoskeletal reorganization. Therefore, our data suggest that one way for prenatal cocaine exposure to exert its influences on offspring

development is mediated by a prolonged PKC activation which consequently increases phosphorylation of downstream substrates. This downstream effect of prenatal cocaine exposure is shown spatially controlled by scaffolding proteins, GRIP as one example.

Collectively, our findings support the hypothesis that prenatal cocaine exposure hyper-activates GRIP-associated PKCs, PKCg and PKC/Mz. The hyper-activated PKCg and PKC/Mz is the result of elevated PDK1 expression and sustained DAG in the membranes resulted from a decreased DGK expression and GRIP-associated DGKs. Earlier reports indicate that excessive activation of PKC in the cortex impairs cognitive functions and increases distractibility and impulsivity (Birnbaum *et al.*, 2004). Our results showing prenatal cocaine exposure leads to hyper-activation of PKC may therefore provide a molecular underpinning for the cognitive impairments observed in animals and humans that have had exposed to cocaine during gestation. Importantly, our data illustrate that normalization of PKC using pseudosubstrate PKC inhibitors may ameliorate the synaptic dysfunction associated with defected AMPARs induced by prenatal cocaine exposure.

Chapter 4. Materials and Methods

Animal Treatment

All animal procedures are in compliance with the National Institutes of Health *Guide for Care Use of Laboratory Animals* and were approved by the City College of New York Animal Care and Use Committee. The pregnant female rats are housed individually in a 12-hr light/dark cycle with free access to food and water. The presence of sperm-positive vagina plug is considered the gestational day (GD) 0. On GD 8, pregnant rats are assigned to receive daily (10am) intra-peritoneal (IP) injections from GD 8-21 of either cocaine HCl, 30 mg/kg in 0.9% saline or saline, 2 ml/kg. Following each injection, these pregnant rats are observed for 1 hour and behavioral abnormalities recorded. While an apparent increase in loco-motor activity was noted in cocaine-treated rats, there were no seizures observed. The progenies are group housed with their mother until they were sacrificed at 21-day of age (P21). Food and water are freely available. They are subjected to the minimum handling associated with routine animal husbandry. Since we did not find gender differences in our previous studies conducted in rabbit and rats (Yablonsky-Alter *et al.*, 2005; Bakshi *et al.*, 2009), both sexes from separate litters were employed in these experiments. Both sexes from separate litters are employed in these experiments. Pups are sacrificed by rapid decapitation. The brain is removed immediately, and dissected on ice.

Organotypic Brain Slice Culture

Frontal cortices were prepared using a McIlwain tissue chopper. Slices were rinsed in ice-cold Kreb's-Ringer and cultured in 20% FBS-, 5% horse serum-supplemented MEM culture medium at 36°C in 5% CO_2 culture hood for 2 days. To compare the effect of M-ZIPand M-GIP to

control M-SCP on PKC-mediated GRIP phosphorylation and GluR2 cellular distribution, slices were rinsed with PBS and incubated for 4 hour in 0.1% FBS containing culture medium. Fresh 0.1% FBS containing medium was then added with 50 μg/ml of M-ZIP, M-GIP, or M-SCP, or vehicle and incubation continued for 60 min at 37˚C. The reaction was terminated with washing. Slices were then incubated in fresh serum-supplemented culture medium. A subset of slices was removed 16 hours later for the planned assays. The same steps of experiments were performed on days 2 and 3.

PDK1 Kinase Activity Assay

Synaptosomes (P2 fraction) were prepared from frontal cortices. Briefly, the animals were decapitated and frontal cortex and different regions of the brain were removed on ice. Tissue fractions were hand homogenized in 10 volumes of ice-cold sucrose buffer (25mM HEPES (PH7.4), 0.1mM EDTA, 0.32M sucrose and inhibitors cocktail) using Teflon-glass homogenizer (10 strokes). After a 5 min 3000 rpm centrifugation at 4°C a pelleted nuclear fraction was removed, and the supernatant was centrifuged for 30 min at 15,000 rpm 4°C to obtain the synaptosome pellet.The synaptosome-enrich P2 fraction was washed twice in 10 volunes of ice-cold Kreb's-Ringer solution (25 mM HEPES, pH 7.4; 118 mM NaCl, 4.8 mM KCl, 25 mM NaHCO3, 1.3 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM glucose, 100 μM ascorbic acid, 50μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor, 0.04 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na2VO4 and 0.5 μl/ml protein phosphatase inhibitor I & II cocktails). The desired fractions were sonicated in immunopresipitation buffer (25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 50 μg/ml leupeptin, 0.2 mM PMSF, 25 μg/ml pepstatin A, 0.01 U/ml

soybean trypsin inhibitor, 5 mM NaF, 1 mM sodium vanadate, 0.5 mM β-glycerophosphate and 0.1% 2-mercaptoethanol) on ice for 5 seconds. The obtained homogenates were solubilized using 0.5% digitonin, 0.2% sodium cholate, 0.5% NP-40 and 0.2% SDS in the presence of cocktails of protease and protein phosphatase inhibitors for 60 min at 4ºC with end-over-end constant shaking. Following centrifugation at 13,200 rpm for 30 min to remove insoluble debris, the obtained lysates were checked for protein concentration. 1 mg of protein was taken for each sample according to the protein concentration and added with IP buffer to make up the volume of 1.2 ml. 5 µl Anti- PDK1 were mixed well and incubated at 4ºC for 30 min followed by adding 50 µl of protein A/G-conjugated agarose beads (Santa Cruz Biotechnology sc-2003) and incubation on shaking table overnight at 4°C.

Next day immunoprecipitates were washed by IP buffer for 2 times and collect the beads. The beads were subjected to kinase assay. Controls were prepared by boiling the beads before kinase assay. 22 µl of reaction buffer (25 mM Tris-HCl, pH 7.5, 10mM MgCl2, 1mM ATP) was added into each sample. 400ng (8 µl) purified SGK was added into each sample as substrate. Samples were incubated in a water bath shaker at 30ºC for 30 minutes. Reaction was terminated by adding 10 µl 4X sample buffer and boiled at 100ºC for 10 minutes. The samples were analyzed by western blotting with gradient gels and the nitrocellulose membranes were incubated with two antibodies (anti-PDK1 and anti-p-SGK) over night. The signals were detected using a chemiluminescent method (Pierce) and visualized by exposing to x-ray film. The films of western blots were analyzed by Image J and Excel.

Quantitative Assay for sn-1, 2-Diacylglycerols

The DAG mass contained in lipid extracts of tissue samples were measured by utilizing standard

E. coli DAG kinase, in the presence of ATP [57], to phosphorylate the DAG to yield [γ-32P] phosphatidic acid(PA), which could then be separated by thin-layer chromatography(TLC). The radioactivity of samples was assessed using scintillation spectrometry (Beckman).The whole tissue assay was prepared using dissected tissue sections. Each tissue section was weighed, and hexane was added x/50(v) to make the tissue concentration equal to 50 mg/ml. After sonication, samples were spun at 50,000×g for 5 min and then supernatants were collected. 200 μl supernatant was taken from each extract. The leftover pellets were first completely dried, then water was added x/50 (v) to form a suspension and protein concentrations were measured by the Bradford method for later correction. Supernatants taken from the samples were dried using nitrogen. Into each dried sample tube, 190 μl reaction buffer (5 mM alfa-cyclodextrin, 100 mM imidizo buffer PH 6.6, 100 mM NaCl, 25 mM MgCl2, 2 mM EGTA) and 10 μl standard DGK were added The reactions were started by adding ATP cold 2.5mM/ hot 0.15ul to each assay for the kinase reaction. The reaction system was incubated at 30ºC for 30 minutes. CHCl3/CH3OH/water (1/1/2ml) was added into each tube to terminate the reaction and separate the phase. The lower chloroform phase was washed with 2 ml of prepared upper phase twice, completely dried and re-dissolved in 100ul of 10% methanol in chloroform, and spotted on a plastic silica thin layer chromatography plate. Plate was air-dried immediately before developing in CHCl3/CH3OH/water/acetic acid (65:45:4:1) solvent system followed by air blow-dry. Lipids were visualized by either exposure of the chromatograms to iodine vapors or by spray reagent []. PA bands were scraped off and the radioactivity assessed by liquid scintillation counter after the addition of 0.4 ml water and 10 ml 5% Omnifluor in toluene: Triton X-100 (4:1 by vol). Blank controls were prepared identically except that tissue extractions were boiled for 5 min before being used.

The synaptosome membranous fractions of 6 pairs of samples were prepared using the protocol of fractionation.The prepared fractions were dried using nitrogen and then homogenized in 0.5 ml hexane to extract the lipids, following the same steps as for the whole tissue assay to measure the DAG amount of each sample.The cold/hot ATP ratio was adjusted to 0.5mM/0.2ul per assay. The results are going by per 1mg membranous protein.

DGK Kinase Activity Assay

DGK isozymes were first purified by immunoprecipitation and their kinase activities were determined by measuring radioactive phosphate incorporation into PA using [r-^{32}P] ATP and exogenous standard DAG as substrates. Synaptosome fractions were prepared first and solubilized by triple detergent: 0.5% NP-40, 0.2% sodium cholate, 0.5% digitonin. Protein concentrations of lysates were checked by Bio-Red. The same amount of protein (1mg) was taken and the volume of each sample was made up with IP buffer for immunoprecipitation, following protocol. Obtained immunoprecipitates were re-suspended in the same reaction buffer as for the measurement of DAG mess and followed up with the same steps as protocol for TLC.

Data Analysis and Statistical Evaluation

The films of western blots were analyzed by Image J and Statistical differences between cocaine and saline contol groups were assessed using the two-tailed Student's t test.

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