PRENATAL COCAINE EXPOSURE AND AMPA RECEPTOR SIGNALING IN THE BRAIN

Thesis

By

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A dissertation submitted to the Graduate faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

PRENATALCOCAINE EXPOSURE AND AMPA RECEPTOR SIGNALING IN THE BRAIN

By

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Prenatal cocaine exposure compromises glutamatergic α-amino 3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPAR)-regulated synaptic transmission, whereas the molecular mechanism underlying AMPAR deficits is unclear. Using brain tissues from 21-day-old *in utero* cocaine-exposed rats, we tested the hypothesis that deficient AMPA transmission in the prenatal cocaine-exposed brain is caused by a reduced synaptic membrane recruitment of AMPARs. Our results show that synaptic membrane expression of AMPAR subunits-GluR1, GluR2 and GluR3 is markedly reduced in the frontal cortex of cocaine-exposed rats. Further, the attenuated synaptic membrane targeting of GluR2/3 in prenatal-cocaine exposed progeny is a result of reduced interaction between GluR2/3 and glutamate receptor interacting protein (GRIP1/2), a PDZ domain protein that mediates the synaptic delivery of GluR2/3. The reduced GRIP – AMPAR interaction is caused by persistent GRIP phosphorylation by protein kinase C (PKC)- and Src tyrosine kinase. To further understand the molecular mechanisms involved in these changes, the role of GRIP-associated protein, GRASP-1, a neuronal rasGEF that interacts with both GRIP and AMPARs in inhibiting synaptic targeting of AMPARs was examined. Our results show an overall increase in the

cytosolic and synaptic membrane levels of GRASP-1 along with an increase in GRIP1/2 and GRASP-1 coupling in the frontal cortex of the prenatal cocaine-exposed brain. We found a reduced GRASP-1 RasGEF activity in the prenatal cocaine-exposed brain followed by an increase in the levels of active/GTP bound small G proteins, RhoA, Rac1/Cdc42 and Rap1 which belong to the Ras superfamily. Moreover, we detected high levels of F-actin in prenatal cocaine exposed progeny which maybe indicative of changes in AMPAR trafficking. These results suggest that change in the phosphorylation state of GRIP not only reduces the synaptic targeting of GluR2/3 but also affects AMPAR trafficking in the prenatal cocaine-exposed brain.

This thesis is dedicated to my Family

To my parents Arun and Saryu Parikh
My uncle Kalapi Parikh who encouraged me to pursue my goal
My life partner Priyam for years of love and support

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This thesis is the end of a five year long journey where, I was accompanied by many people who have contributed in different ways to make this possible. I owe my gratitude to all those who have made this dream achievable for me.

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TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
FCX	Frontal Cortex
GluR	Glutamate receptor
NMDA	N-methyl D-Aspartate
PDZ	Postsynaptic density 95/Discs large/zona occludens-1
GRIP1/2	Glutamate receptor interacting protein
ABP	AMPA receptor binding protein
PKC	Protein Kinase C
GRASP-1	GRIP interacting protein
GEF	Guanine nucleotide exchange factor
PICK	Protein interacting with C Kinase
LTD	Long term potentation
LTP	Long term depression
PBS	Phosphate-buffered saline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphotidylserine
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline

CHAPTER-1

Introduction

Glutamate Receptors

Glutamate receptors mediate the majority of excitatory synaptic transmission in the central nervous system (CNS). Glutamate receptors are critical in a wide variety of neuronal developmental and maturational processes such as cell proliferation, neuronal migration, synaptic plasticity and cell death. Based on their pharmacological and structural similarities, glutamate receptors are divided into two classes: ionotropic receptors and metabotropic receptors. The main classes of ionotropic receptors are N-methyl d-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), Kinate receptors and Delta receptors (figure1.1). Ionotropic glutamate receptors are multimeric assemblies of four or five subunits which share a common basic structure. All the subunits consist of an extracellular amino (N) terminus, three transmembrane spanning domains and a reentrant loop which lies close to the cytoplasmic C terminal domain. Depending on their subunit composition, the ionotropic glutamate receptor channels can flux Na^+, K^+ and/or Ca^{2+} ions. Metabotropic glutamate receptors (mGluRs) are G protein coupled receptors which are further subdivided into three groups based on their primary sequence and pharmacological properties. Group I mGluRs activate phospholipase C (PLC) and intracellular calcium signaling whereas the group II and III negatively couple to adenylyl cyclase.

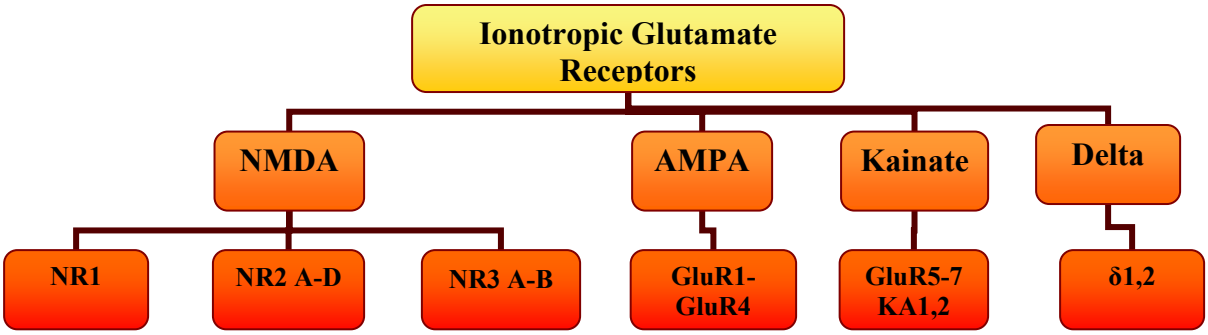

Figure. 1.1: Subtypes of Ionotropic glutamate receptor. NMDA receptors are composed of assemblies of NR1-NR3 subunits. The non-NMDA receptors are further classified into AMPA composed of combinations of GluR1-GluR4 subunits, Kainate composed of GluR5-GluR7 and KA1-2, and Delta (δ1, 2).

AMPA Receptors

Among various glutamatergic receptors, AMPARs are the primary mediator of the fast excitatory synaptic transmission in the CNS. Glutamate binding to AMPAR produces a rapid depolarization which decays rapidly, hence AMPA are also known as fast conducting channels. In the CNS, AMPARs not only maintain the basal excitatory synaptic transmission but also have been implicated in inducing activity-dependent synaptic plasticity underlying long term potentiation (LTP) and long term depression (LTD). AMPARs are cation channels composed of different combinations of GluR1-GluR4 or GluRA- GluRD subunits which form both homomeric and heteromeric channels (Hollmann & Heinemann, 1994; Roche et al., 1994). All the subunits share 68-73% sequence identity and are of similar size (~900 amino acids) (Hollmann & Heinemann, 1994). Typically, AMPAR subunits have an extracellular N (amino) terminal domain (NTD), four hydrophobic segments and a cytoplasmic C terminal domain (figure 1.2). The large extracellular NTD comprises of 400 amino acids, followed

by a region (S1) which is composed of 200 amino acid and is homologous to the glutamine binding protein (QBP) from bacteria (figure 1.2) (Bredt & Nicoll, 2003). The second extracellular region (S2) (figure 1.2) also shows homology to bacterial amino acid binding protein and is required along with S1 for glutamate binding. The topology of the subunits shows that the NTD is followed by the first membrane spanning domain and the second hydrophobic segment forms a hairpin-like reentrant loop on the cytoplasmic side (figure 1.2) (Hollmann et al., 1994). The second segment forms the channel pore and is followed by two transmembrane spanning domains and a cytoplasmic (carboxy/C terminus) tail (figure 1.2).

GluR1-GluR4 mRNAs are present as early as embryonic day 10 in the CNS as well as in the peripheral nervous system, however, the proteins are expressed postnatally (Bettler et al., 1990; Hollmann &Heinemann, 1994). The levels of GluR1, GluR2 and GluR3 subunits increase gradually during postnatal development and stabilize between postnatal days 14- 20 whereas the expression of GluR4-subunit is mainly restricted to the first postnatal week (Zhu et al., 2000). AMPARs are expressed as different combinations of GluR1-GluR3 subunits in mature neurons (Wenthold et al., 1996). There is a notable difference in the expression of subunits in different brain areas. In the adult, GluR1-GluR3 mRNAs are expressed predominantly in the pyramidal and granule layers of the hippocampus and the Purkinje and granule cell layers of the cerebellum. In the neocortex, GluR2 mRNA labeling is very prominent and exhibits clear lamination whereas GluR1 and GluR3 show little lamination (Pellegrini-Giampietro et al., 1991). Again, in the stratium GluR2 mRNA are more abundant than other two subunits, whereas all the three

mRNAs are expressed at lower levels in the thalamus, hypothalamus and lower brain stem (Pellegrini-Giampietro et al., 1991).

The second extracellular region (S2) of all AMPAR subunits is alternatively spliced to form either a flip or flop variant which results in differential kinetic properties of the channel (figure 1.2) (Sommer et al., 1990). Monyer and coworkers (1991) have shown that during the embryonic stages all the four subunits are in the flip form, whereas postnatally they are in the flop form. Receptors with the flip variant are known to allow more current entry into the cells then receptors containing flop sequences which may be important for synapse formation during early development and may play a role in the maintenance of LTP (Monyer et al., 1991; Sommer et al., 1990). In response to glutamate, the flop variant desensitizes more rapidly than the flip form. The GluR2 subunit is particularly interesting because it undergoes post-transcriptional RNA editing where an arginine (R) replaces a glutamine (Q) residue at a Q/R site in transmembrane domain II (Sommer et al., 1991). The receptors containing the edited form of GluR2 (R) subunits are impermeable to Ca^{2+} (Sommer et al., 1991). Although the majority of the GluR2 subunits in the adult CNS are the GluR2 (R) variant, the prevalent presence of GluR2 in the CNS indicates that altered Ca^{2+} permeability through GluR2 is functionally significant.

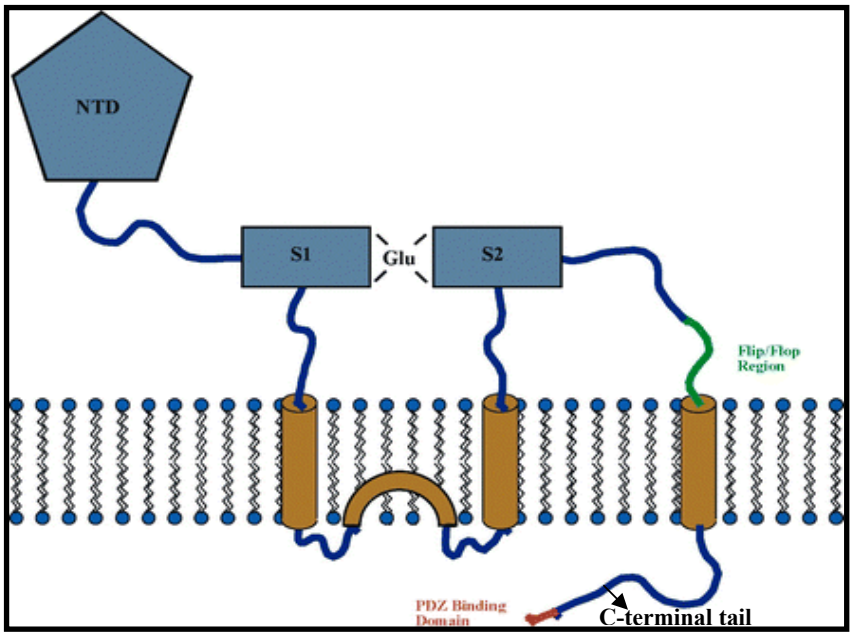

Figure 1.2: Schematic representation of AMPAR subunit (GluR). AMPAR subunits have an extracellular amino/N terminal domain (NTD) and a cytoplasmic C-terminal tail. Three of the four hydrophobic segments span the membrane and one dips into the membrane to form the channel pore. The C-terminal of short tailed subunits has a PDZ domain binding site whereas the NTD forms the glutamate binding site (S1 and S2). Modified from Bredt & Nicoll, 2003.

An important functional distinction among the AMPAR subunits is the length of their cytoplasmic C-terminal tail, which controls the trafficking of AMPARs (Passafaro et al., 2001; Shi et al., 2001). GluR1 subunit always has a long tail and GluR3 has a short one whereas the tails of GluR2 and GluR4 are alternatively spliced so that these subunits can either have a long or a short tail (Kohler et al., 1994). Studies have shown that activity-dependent synaptic enhancement recruits AMPARs with long cytoplasmic tails (e.g., GluR1, GluR2L and/ GluR4) (Hayashi et al., 2000; Zhu et al., 2000), whereas AMPARs with only short cytoplasmic tails (e.g., GluR2 or GluR3) cycle continuously between cytosol and synaptic sites in an activity-independent manner (Passafaro et al., 2001; Shi et al., 2001). More importantly, the number of GluR2/3-containing AMPARs at synapses can be increased in response to synaptic stimulation but reduced following

synaptic depression (Kim et al., 2001; Lin et al., 2000; Luscher et al., 1999; Luthi et al., 1999; Shi et al., 2001; Snyder et al., 2001). 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 synaptic delivery of AMPARs containing GluR1 and GluR4 subunits is also dependent on the activation of NMDA receptor (Shi et al., 1999). In response to NMDA receptor stimulation and activation of calcium calmodulin dependent kinaseII (CaMKII), GluR1 subunit recruits AMPARs to the synapse resulting in synaptic potentiation. Numerous studies have established that in the hippocampus GluR1 containing AMPARs are delivered to the synaptic membrane during activity dependent synaptic potentiation such as LTP. Shi and colleagues (2001) have proposed that AMPAR subunits are recruited to the postsynaptic membrane in the order of GluR1 dominating over GluR2 and GluR2 precedes GluR3. Therefore, the synaptic membrane recruitment of GluR1 containing AMPAR subunits determines the strength of basal synaptic transmission and regulates synaptic plasticity. Different studies have shown that targeted deletion of GluR1 gene results in an impaired hipppocampal LTP (Zamanillo et al., 1999) while GluR2 deficient mice show enhanced LTP (Jai et al., 1996). This notion is further strengthened by studies showing dramatic reduction in basal synaptic transmission in GluR2 and GluR3 double knockouts (Meng et al., 2003). Mice expressing functionally impaired GluR2 show increased mortality and those survived exhibit reduced exploration and impaired motor coordination (Jia et al., 1996). It has also been shown that certain mutations in GluR2 alters synaptic transmission and influence the severity of neurological dysfunctions, epileptic threshold and dendritic architecture

defects (Jia et al., 1996; Feldmeyer et al., 1999). Altogether, these studies indicate that alteration in the synaptic recruitment of GluR2 may severely impact the dendritic integrity and basal synaptic transmission leading to brain dysfunctions.

Interaction of AMPAR with PDZ domain containing proteins

AMPARs lack motor domains; therefore, their subunit constituents interact through the cytoplasmic C-terminus tail with specific proteins called the postsynaptic density 95/Discs large/zona occludens-1 (PDZ) domain, containing synaptic scaffolding proteins. PDZ domains are motifs of about 90 amino acids that mostly bind to the C-terminal region of various interacting partners and play a general role in scaffolding membrane proteins (figure 1.2 and 1.3). The PDZ domain containing synaptic scaffolding proteins mediates the interaction of AMPARs with other proteins, which in turn interact with cytoskeletal elements/actin filament assisting in the trafficking of AMPARs. An example of a typical PDZ domain protein is post-synaptic density-95 (PSD-95), which is an NMDA receptor interacting partner that links the receptor to downstream signaling enzymes. Similarly, the interaction between PDZ domain proteins and the C-termini of AMPAR subunits GluR2 and GluR3 is pivotal in the regulation of receptor targeting to excitatory synapses. The GluR1 subunit contains a tri-peptide sequence which binds to different PDZ domain partner SAP-97 (Leonard et al., 1998). The interaction between GluR1/SAP-97 regulates the synaptic trafficking of AMPARs. The GluR2 -GluR3 subunits associated PDZ domain proteins include Glutamate Receptor Interacting Protein (GRIP1/2) (Dong et al., 1997) (fig 1.3), AMPAR Binding Protein (ABP) (Srivastava et al., 1998) and Protein Interacting with C Kinase-1 (PICK) (Dev at al., 1999 and Xia et al.,

1999) (figure 1.2). The C-terminal short tails of AMPARs also interact with a non-PDZ protein, N-ethylmaleimide-sensitive fusion protein (NSF) (figure 1.3), a multimeric ATPase that plays an essential role in membrane fusion and trafficking of GluR2 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). The NSF binding site is in the center of the C-terminal tail upstream of the PDZ binding site.

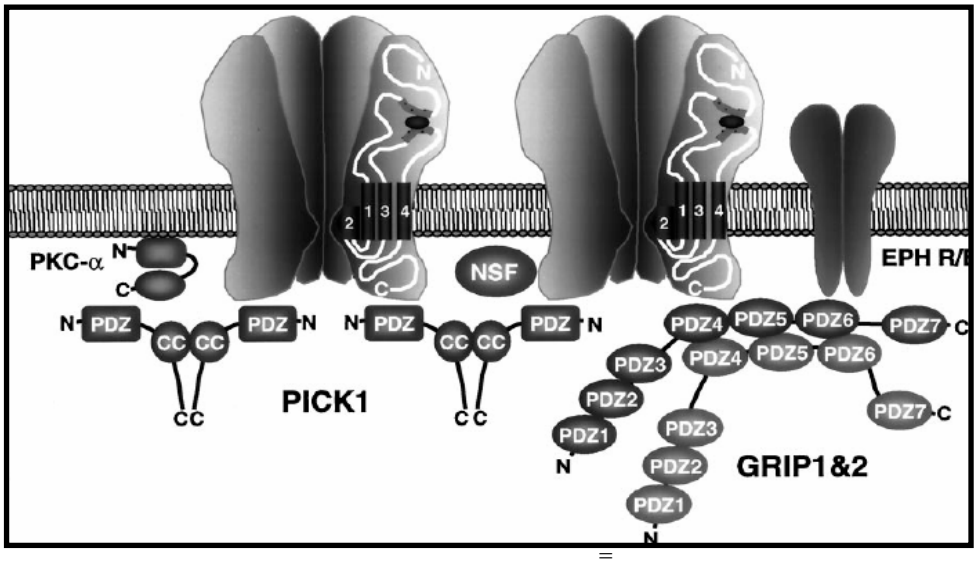

Figure 1.3: AMPAR GluR2 and GluR3 binding proteins. AMPAR subunits containing short C-terminal tail, GluR2 and GluR3, interact via their C-terminal tail with PDZ domain containing proteins GRIP1&2 and PICK1 and also non PDZ protein NSF. Modified from Dong et al., 1999.

Interaction of AMPAR with synaptic scaffolding protein GRIP

GRIP1 and GRIP2 are neuronal proteins which are highly homologous within the PDZ domain and are enriched in post-synaptic densities (PSD) (Dong et al., 1999). GRIP1 is expressed in the rat brain early during embryonic development, whereas GRIP2 expression is detected postnatally, however their levels stablilize around postnatal day 8 and 14 respectively (Dong et al., 1999). On Western blots, GRIP protein appears as a

heterogeneous band (approximately 130 kilodaltons) that is expressed in widespread brain regions and throughout postnatal development. Biochemical studies reveal that GRIP is largely membrane-associated and enriched in the PSD, though not as highly concentrated as PSD-95. By immunohistochemistry, GRIP is distributed in a somatodendritic pattern in neurons of the adult rat brain, with especially prominent expression in a subset of interneurons (Dong et al., 1999). GRIP contains seven PDZ domains and interacts with the C terminus of GluR2 and GluR3 via fourth and fifth PDZ domain (figure 1.3) (Dong et al., 1997). Both GluR2 and GluR3 subunits interact with GRIP1/ABP family of proteins via C terminal –SVKI sequence which is a type II PDZ binding site (Dong et al., 1997; Srivastava et al., 1998; Dong et al., 1999; Bredt & Nicoll., 2003). Mutagenesis studies in hippocampal neurons have shown that a GluR2 mutant lacking PDZ binding site can still be transported to the synaptic membrane, however, the surface accumulation of the mutant GluR2 is drastically reduced than the wild type over time (Osten et al., 2000). Furthermore, these reports also show that blocking the binding of GRIP/ABP to GluR2 by mutating a single residue in GluR2 significantly reduces the synaptic membrane expression of GluR2 subunit. Other studies have shown that GluR2/3 interaction with GRIP regulates that synaptic targeting of this receptor pool and LTD (Chung et al., 2000; Kim et al., 2001). These studies indicate that GRIP/ABP in the synaptic membrane interacts intimately with AMPAR-GluR2 subunits. In addition, the PDZ domain protein PICK is also involved in regulating internalization of AMPAR subunits, GluR2 and GluR3 (Dev at al., 1999 and Xia et al., 1999). While GRIP1-GluR2/3 interaction mobilizes the receptor to the synaptic membrane, GluR2/3

association with PICK1 results in internalization and thus removal of AMPARs from the synaptic membrane (figure 1.4) (Chung et al., 2000; Perez et al., 2001; Kim et al., 2001).

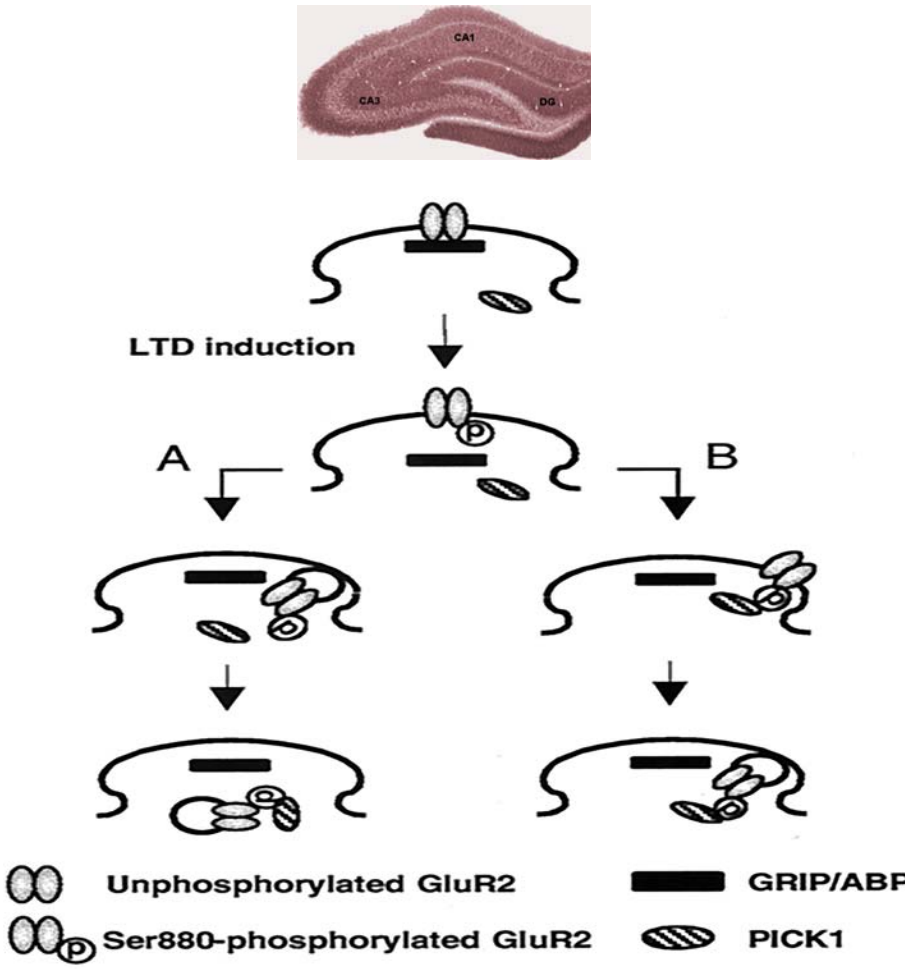

Figure 1.4: Model for the regulation of AMPAR internalization via phosphorylation of subunits. In the basal state, AMPAR containing GluR2/3 are stabilized at the synaptic membrane by their interaction with GRIP/ABP. (A) LTD induction in the hippocampus increases the phosphorylation of GluR2 at Ser^{880} which disrupts the GluR2-GRIP interaction, leading to receptor internalization. PICK1 binds to the internalized $pSer^{880}$ GluR2 and stabilizes the subunit. (B) On the other hand, PICK1 may trigger the internalization of $pSer^{880}$ GluR2. Modified from Kim et al., 2001.

Phosphorylation of AMPAR subunits regulates the synaptic localization and its interaction with the PDZ domain-containing proteins. In this regard, Esteban and colleagues (2003) have shown that in organotypic hippocampal slices, PKA

phosphorylation of GluR1 and GluR4 directly controls the synaptic incorporation of AMPARs. Similarly, PKC mediated phosphorylation of GluR2 subunit at Ser^{880} site on the C terminal PDZ domain binding sequence differentially regulates its interaction with GRIP and PICK (figure 1.4) (Chung et al., 2000; Matsuda et al., 1999; 2000; Kim et al., 2001). These reports show that *in vitro* Ser^{880} phosphorylation of GluR2 significantly reduces its interaction with GRIP1 but increases the interaction with PICK and induces the internalization of GluR2 (figure 1.4) (Kim et al., 2001). Studies have also shown that blockade of GRIP1-GluR2 interactions prevents potentiation of synaptic responses (Sheng, 2001; Li et al., 1999). Collectively, these studies support the notion that GluR2-GRIP1 interaction is involved in the recruitment of functional AMPARs to the synapses, which in turn determines the efficacy of excitatory synaptic transmission. Hence, the interaction of AMAPRs with the PDZ domain containing proteins determines the strength of synaptic transmission and therefore any changes in these interactions may lead to modifications in synaptic plasticity.

GRIP associated protein GRASP-1 and trafficking of AMPAR

In addition to associating with GluR2/3, GRIP1/2 also serves as an adapter to other proteins including ephrin receptor tyrosine kinases (Eph), ephrin-B ligands (Torres et al., 1998; Bruckner et al., 1999), scaffold protein Liprin-α (Wyszynski et al., 2002) and GRIP associated protein, GRASP-1, a neuronal RasGEF (guanine nucleotide exchange factor), (Ye et al., 2000) that has been implicated in the regulation of membrane protein trafficking and actin cytoskeleton dynamics. A recent study reports that GRIP1 is required for the formation and growth of dendrites in developing neurons and for the maintenance of dendrites in mature neurons, a function regulated via EphB2 receptor

(Hoogenraad et al., 2005). Furthermore, it has been shown that GRIP1 mutation results in embryonic lethality as these mutants have disregulation in epithelial-mesenchymal interaction during morphogenesis (Takamiya et al., 2004). This suggests that GRIP1 is not only involved in receptor trafficking but is also critical in the development, maturation and health of dendrites. The GRIP binding partner, GRASP-1, a neuron-specific RasGEF, is particularly interesting because it interacts with both GRIP1 and GluR2 at different sites (Ye et al., 2000). The GRASP-1, with its RasGEF domain in the N terminal region, interacts with the seventh PDZ domain of GRIP located at the C terminal region. GRASP-1 forms a complex with GRIP and GluR2 *in vivo*, and the overexpression of GRASP-1 inhibits the synaptic targeting of AMPARs in cultured neurons (Ye et al., 2000). GRASP-1 has been shown to have RasGEF activity (Ye et al., 2000) and RasGEFs play a critical role in receptor trafficking, which makes it important to characterize the role of GRASP-1 in AMPAR trafficking. Ras activates small G proteins that belong to the Ras superfamily of small GTP binding proteins which act as effectors in regulating the functions of Ras.

Small GTP binding proteins like RhoA, Rac1/Cdc42 and Rap1 that belong to the Ras superfamily are known to regulate receptor trafficking by influencing actin dynamics. RhoA and Rac1/Cdc42 belong to the Rho subfamily whereas Rap1 belongs to the Ras subfamily. The role of small GTP binding proteins in controlling actin cytoskeletal reorganization and cell morphology is well established. These proteins act as molecular switches that cycle from an inactive GDP bound state to an active GTP bound state which controls many cellular functions like trafficking, cytoskeletal reorganization and cell motility. In the CNS, proteins belonging to the Rho family (Rho, Rac and

Cdc42) are expressed in hippocampal pyramidal neurons and dentate granule cells, the neocortex and other regions (Olenik et al., 1997). It has been shown that Ras and Rap regulate AMPAR trafficking during LTP and LTD (Zhu et al., 2002), Rac1/Cdc42 induces clustering of AMPARs during spinogenesis (Wiens et al., 2005) and RhoA regulates actin polymerization in dendritic spines (Schubert et al., 2006). Collectively, these data suggest that GRASP-1 being a neuronal RasGEF may regulate AMPAR trafficking by modulating actin-dependent movement of AMPARs through regulating the activity of Ras like small G proteins such as Rap1, Rac1/Cdc42 and RhoA.

The role of glutmatergic receptors in cocaine abuse

In the adult brain cocaine exposure profoundly affects glutamatergic synaptic transmission and plasticity in diverse brain areas, but the effects of prenatal cocaine exposure on glutamatergic transmission remains largely elusive. Among various glutamatergic receptors, AMPARs appear to be sensitive to cocaine perturbation. This is supported by the data illustrating that pharmacological inhibition of the AMPARs in reward-relevant brain regions like the nucleus accumbens (NAc) and ventral tegmental area (VTA) attenuates cocaine addiction (Kaddis et al., 1995; Harris & Aston-Jones, 2003; Backstrom and Hyytia, 2003). In addition, 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). In adult rat, extinction training following cocaine self-administration increases the expression of AMPAR subunits GluR1 and GluR2/3 in the NAc shell. Moreover, the overexpression of these AMPAR subunits increases response to extinction training (Sutton et al., 2003). Ungless et al (2001) report an increase in AMPA to NMDA ratio, an indication of LTP, in the VTA region of mice

exposed to single cocaine injection. Recently, some studies have shown that psychostimulants like cocaine and amphetamine in the adult modify the phosphorylation state of NMDA receptor subunits (Loftis & Janowsky 2002) and AMPAR GluR1 (Snyder et al., 2000; Chao et al., 2002) which in turn alter the synaptic membrane expression and function of these receptors. Although these data show a profound influence of cocaine exposure on AMPARs, the effect of prenatal cocaine exposure on AMPAR neurotransmission and the underlying molecular mechanism remains elusive.

Prenatal cocaine exposure and glutamate receptors

Prenatal cocaine exposure is known to have a long-lasting detrimental effect on the structure and function of the brain in a number of ways. Studies in both human and animal models of prenatal cocaine exposure show permanent deficits in the brain development. Prenatal exposure to cocaine in rabbits impairs motor function, associative learning, discrimination learning and permanently alters dendritic length and shape of pyramidal neurons in the cerebral cortex (Romano et al., 1995;1996 ; Jones et al., 1996; Stanwood et al.,2001). Collectively, all these studies indicate that alterations in the brain glutamatergic systems resulted from prenatal cocaine exposure play a pivotal role in mediating deficit in learning and cognitive processes that may ultimately lead to abnormal responsiveness to stressful conditions.

Accumulated evidence points to the role of dopaminergic pathways in prenatal cocaine exposure. Prenatal cocaine exposure in rabbits has been shown to disrupt dopaminergic receptor 1(D1) coupling to its associated $G_{s/olf}$ protein in the cortex and striatum (Wang et al., 1995; Friedman et al., 1996; Jones et al., 2001). The pathways that

are known to be involved in mediating the reward aspect of cocaine include the mesolimbic, mesocortical and striatal pathways that are enriched with both dopaminergic and glutamatergic innervation which is indicative of cross talk between these systems. In support of this notion, dopaminergic neurotransmission, the anchor of reward circuit, was shown to be regulated by various glutamatergic systems (Kalivas & Volkow 2005). Cornish and Kalivas (2000) demonstrate that glutamate (AMPA), and not dopamine transmission in the NAc, is a primary mediator of cocaine-induced reinstatement of drug-seeking behavior and AMPAR antagonists effectively blocked reinstatement. Moreover, Gao and coworkers (2006) report that activation of D1 dopamine receptors increases the surface expression of GluR1 containing AMPARs facilitating their synaptic incorporation. Although the above-mentioned studies suggest the role of glutamate receptors in cocaine abuse, to date there are no studies examining the role of glutamate receptors in the prenatal cocaine exposed the brain. The glutamatergic and dopaminergic pathways converge at the reward relevant areas in the brain which mediate the effects of drugs of abuse. Moreover, glutamate receptors are the key regulators of neuronal migration, differentiation, neurogenesis that determines final neuron number in the neocortex during development, it is therefore important to characterize the role of glutamate receptors in prenatal cocaine exposed brain. Therefore, in this thesis research, I focus my effort on investigating the changes in glutmatergic AMPARs and the molecular mechanism underlying those abnormalities in the prenatal cocaine-exposed brain.

Scope of this thesis

In this thesis we focused primarily on the frontal cortical region of prenatal cocaine exposed rats because the frontal cortex serves as an interface between the

corticolimbic regions which regulate mood and rewarding properties of drugs of abuse as well as cognitive processes such as learning and memory. Moreover, there are abundant glutamatergic receptors in the frontal cortex region and the cognitive deficits seen in both the animal model and in human prenatal cocaine exposure can be linked to this brain area. Using frontal cortices from 21-day-old rats that have had exposure to cocaine *in utero*, we tested the hypothesis that ***deficient AMPAR transmission in prenatal cocaine-exposed brain is caused by a reduced synaptic AMPAR recruitment***. This hypothesis is based on the results from the preliminary studies conducted in the frontal cortices from 21-day-old prenatal saline- and cocaine-exposed rats. The data demonstrate that prenatal cocaine exposure (1) abolishes NMDA-induced synaptic recruitment of GluR1, and (2) markedly reduces the synaptic recruitment of AMPAR GluR2 and GluR3 subunits. Our studies show that a reduced GluR2/3 synaptic recruitment in the frontal cortices of prenatal cocaine-exposed rats is the result of a markedly attenuated interaction between GluR2/3 and its binding partner GRIP, an essential step in the constitutive recycling of GluR2- and/or GluR3-containing AMPAR to the synaptic membrane. We show that PKC and Src mediated phosphorylation of GRIP is one of the mechanism responsible for a reduced GluR2/3 and GRIP interaction as we see an increased translocation of typical and atypical isoforms of PKC in the prenatal cocaine exposed brain. To further fully elucidate the molecular mechanisms involved in the trafficking of GluR2/3 containing AMPARs we examine the role of GRIP-associated protein GRASP-1, a neuronal rasGEF that interacts with GRIP and AMPAR complexes in inhibiting synaptic targeting of AMPARs. A previous study has shown that an increased GRASP-1 in the synaptic membrane interferes with the synaptic targeting of AMPARs (Ye et al., 2000). Our

results show an over all increase in the levels of GRASP-1 along with an increase in the interaction between GRIP1/2 and GRASP-1 in the frontal cortex of prenatal cocaine exposed animals. Together with the data illustrating that prenatal cocaine exposure increases actin polymerization-regulating active ras-like G proteins, our data suggest that an enhanced GRASP-1 and GRIP1/2 coupling in the prenatal cocaine-exposed brain is also responsible for the reduced synaptic targeting and actin-dependent trafficking of GluR2/3-containing AMPARs.

CHAPTER-2

Materials and Methods

LIST OF MATERIALS.

Cocaine HCL was obtained from Sigma (St Louis, Mo, USA).

Enzymes

Recombinant γPKC and c-Src was obtained from Cal-biochem (San Diego, CA, USA)

ATP, GDP, [^{3}H] GDP, recombinant Src and H-ras were obtained from Sigma (St Louis, MO, USA).

Kits

Seize-X immunoprecipitatation kit was obtained from PIERCE (Rockford, IL, USA). Rho Activation Assay Kit, Rac/cdc42 Assay Reagent and Rap1 Activation Assay Kit were obtained from Upstate technologies (Charlottesville, VA, USA).

List of antibodies and Probes

The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA): GluR1, GluR2, GluR3, GRIP1, GRIP2, GRASP-1, NSF, β-actin, β-tubulin, Caspase 3, pSer^{880}GluR2, pSer^{881}GluR3, anti phospho threonine, Actin, RhoA, Rap1, Cdc42/Rac1 and HRP conjugated secondary antibodies. PICK1 and ABP were obtained from Chemicon (Temecula, CA, USA). Anti phospho serine and anti phospho threonine were obtained from Sigma (St Louis, MO, USA). Rhodamine-conjugated phalloidin fluorescence probe was obtained from Molecular Probes (Eugene, OR, USA).

METHODS

Animal Treatment

Pathogen-free, 10-week-old male and female Sprague-Dawley rats weighing approximately 200-215g (Taconic, Germantown, NY) were housed individually in a 12 hr light/dark cycle with free access to food and water. All animal procedures were 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. A pair of 10-week-old male and female rats was placed in a cage overnight. In a given experiment set, we used 4 mating pairs. The presence of sperm-positive vagina plug is considered the gestational day (GD) 0. The pregnant female rats were housed individually without disturbance other than the daily injection with cocaine or saline. On GD 1, pregnant dams were assigned to either the cocaine group that received cocaine HCl (Sigma), (30 mg/kg in 0.9% saline injection) subcutaneously (sc), daily from GD 1-21 or saline group that received saline, 2 ml/kg, sc injection daily from GD 1-21. The animals were injected daily between 9-10 AM. Following each injection, these pregnant rats were observed for 1 hr and behavioral abnormalities were recorded. There was an apparent increase in locomotor activity in cocaine-treated rats. To minimize skin lesions and tissue necrosis in the cocaine-injected rats the injection sites were rotated over different sites on the animal's back. The cocaine injection did not affect litter size or body weight of the pups. We also did not find any correlation between the magnitude of tissue necrosis and litter size or body weight of the pups. Importantly, the dose of cocaine used in this study did not induce seizure.

The progenies were group housed with their mother until they were sacrificed at 21-days of age. Food and water were freely available. They were 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 (Wang et al., 1995; Jones et al., 2001; Yablonsky-Alter et al., 2005) both sexes from separate litters were employed in these experiments. At the day of experiment, the pups were sacrificed by rapid decapitation and the brain was removed immediately and frontal cortices were dissected on ice.

Preparation of Synaptosomes & fractionation

Synaptosomes (P2 fraction) were prepared from frontal cortices as previously described with few modifications (Wang et al., 1994; Wang et al., 1999). Briefly, the animals were decapitated, FCX and different regions of the brain were removed on ice. Frontal cortical slices (300μm x 300 μm, 3mm thick) were suspended in Kreb's Ringer solution (25 mM HEPES, pH 7.4; 118 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 100 μM ascorbic acid, 50 μg/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 Na_2VO_4 and 0.5 μl/ml protein phosphatase inhibitor I & II cocktails) (Sigma, St. Louis, MO). The slices were homogenized in homogenization medium containing 0.32M sucrose, 0.1mM EDTA, and 25mM HEPES, pH7.4 using Teflon-glass homogenizer (10 strokes). After a 10 min 1000 x g centrifugation at 4°C a pelleted nuclear fraction was removed, and the supernatant was centrifuged for 15 min at 25,000 x g 4°C to yield a

crude cytosol and synaptosome pellet. To further purify the synaptosomal factions, the synaptosome-enrich P2 fraction was washed twice in 5 ml of ice-cold Kreb's-Ringer solution. To obtain cytosolic and membranous fractions of the synaptosomes, the washed synaptosomes were sonicated for 10 sec on ice in 0.5 ml hypo-tonic homogenization solution (25 mM HEPES, pH 7.4; 12 mM NaCl, 0.48 mM KCl, 2.5 mM $NaHCO_3$, 0.13 mM $CaCl_2$, 0.12 mM $MgSO_4$, 0.12 mM KH_2PO_4, 1 mM glucose, 10 μM ascorbic acid, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor, 0.04 mM PMSF and 0.1 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na_2VO_4 and 0.5 μl/ml protein phosphatase inhibitor I & II cocktails). The samples were then centrifuged at 50,000 x g for 30 min. The resultant supernatant was taken as a cytosolic fraction and the synaptic membrane pellet was resuspended in 0.5 ml of hypo-tonic solution. Protein concentrations of the synaptic membranes were determined using the Bradford method (Bio-Rad) and then solubilized by boiling for 5 min in 6X sample preparation buffer.

Immunoaffinity purification of native GluR2, GluR3, GRIP1 & GRIP2

To isolate native GluR2, GluR3, GRIP1 and GRIP2, the frontal cortices of P21 prenatally cocaine- or saline-exposed rats were homogenized in hypo-tonic homogenization solution described above. 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 20 min at 25ºC followed by 60 min at 4ºC with end-over-end constant shaking. Following centrifugation to remove insoluble debris, the obtained brain lysate was diluted 5-fold with immunoprecipitation buffer (25 mM Na-HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA,

50 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor, 0.04 mM PMSF and 0.1 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na_2VO_4 and 0.5 μl/ml protein phosphatase inhibitor I & II cocktails) and GRIP1, GluR2 and GluR3 were individually purified using an immunoaffinity column (Seize-X immunoprecipitatation kit, PIERCE) with covalently immobilized antibodies directed against GluR2, GluR3, GRIP1 and GRIP2 according to manufacturer's instruction. GluR2, GluR3, GRIP1 and GRIP2 were then eluted twice each with 90 μl antigen elution buffer (PIERCE). The resultant elutes were combined and then neutralized immediately with 20 μl 1.5M Tris, pH8.8 and concentrated to 100 μl passing through 10-KDa cut-off filter (Cole-Palmer). Protein concentrations were determined using the Bradford method (Bio-Rad).

***In vitro* determination of GluR2– GRIP1 interaction and immunoprecipitation**

To control GRIP1 phosphorylation state, native GRIP1 proteins (10 μg) purified from frontal cortices of 21-day-old prenatally saline- and cocaine-exposed rats were incubated with 100 μg/ml alkaline phosphatase (Sigma) in Tris, pH8.0, 130 mM NaCl, protease and phosphatase inhibitors at 30ºC for 20 min (total incubation volume 100 μl). The phosphatase activity was terminated by 10 mM NaF/ 1 mM Na_3VO_4. To achieve PKC- or Src-mediated phosphorylation of GRIP1, alkaline phosphatase-dephosphorylated GRIP1 (5 μg) was incubated with 0.5 μg/ml recombinant γPKC (Cal-Biochem), 20 μg phosphotidylserine, and 100 nM PMA or 10μl/ml recombinant Src (Cal-Biochem) in the presence of 30 μM ATP in Kreb's-Ringer at 30ºC for 10 min (total incubation volume 125 μl). The actions of PKC and Src were terminated by addition of 1 μM celestrine and PP1, respectively. One-half of GRIP1 solution containing 5 μg) was

immediately solubilized by adding 6X sample preparation buffer and boiling for 5 min for analysis of phosphor-serine and -tyrosine levels by Western blotting. Purified brain GluR2 (5 μg) from prenatally saline- and cocaine-exposed rats were individually added to 5 μg of GRIP1 with different phospohorylation states and incubated in 100 μg/ml brain phospholipids, 1% bovine serum albumin (BSA)-containing Kreb's-Ringer at 30°C for 30 min with constant end-over-end shaking. The GRIP1-associated GluR2 was then isolated along with GRIP1 by 20 μl immobilized anti-GRIP1 conjugated protein A-agarose beads and measured using Western blot with anti-GluR2. The obtained blots were screened for phosphoserine first using anti-phosphoserine, stripped and re-probed twice sequentially with anti-phosphotyrosine and anti-GRIP1. The signals were detected using a chemiluminescent method (Pierce) and visualized by exposing to x-ray film.

To assess PICK-GluR2/3 interaction, immunoaffinity-purified PICK1 (5μg) and GluR2/3 (5 μg each) were incubated for 15 min at 30°C in Kerb's-Ringer containing protease and protein phosphatase inhibitors. PICK1 and its associated GluR2/3 were immunoprecipitated with immobilized anti-PICK1 with protein-A/G beads (20 μl). The GluR2 or GluR3 contents in the resultant immunoprecipitate were determined by Western blot with anti-GluR2 or -GluR3.

Western Blotting

To determine the interaction between GluR2 or GluR3 and GRIP1, cytosolic and membranous fractions of frontal cortices or anti-GRIP1 immunoprecipitates were boiled for 5 minutes in 100 μl PAGE sample buffer and then size fractionated on 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and Western blotting was

performed with antibodies for GluR1 (SC-13152), GluR2 (SC-7610), GluR3 (SC-7613), pGluR2/3 (Ser880-Ser891) (SC-17088), phosphotyrosine (SC-508), phosphoserine (Sigma,P3430), phosphothreonine (Sigma, P3555). The blots were stripped and re-probed with anti- GRIP1 (SC-17641).

To measure the expression levels of GluR2, GluR3, GRIP1, GRIP2 (SC- 15477), PICK1, ABP and NSF Western blotting, protein extracts prepared as above were size fractionated on 7.5% or 10% SDS-PAGE based on their apparent molecular masses and Western blotting was performed using antibodies against GluR2, GluR3, GRIP1, GRIP2, PICK1, ABP, NSF,. The blots were stripped and re-probed with anti-β-actin to normalize for sample loading.

To measure cellular distribution of GluR2, GluR3, GRASP-1 and various PKC isozymes, particulate and cytosolic fractions of synaptosomes of prenatally cocaine- and saline-exposed P21 rats were prepared as described above. Following solubilization by boiling in sample preparation buffer for 5 min, fifty μg of cytosolic and membranous proteins were size-fractionated on 7.5% or 10% SDS-PAGE based on their apparent molecular weights. The level of GluR2, GluR3, GRASP-1 and various PKC isozymes were determined by Western blotting with antibodies directed against GluR2, GluR3, GRASP-1 and various PKC isozymes including PKCα (SC- 208), PKCβ (SC-209), PKCγ (SC-211), PKCδ (SC-937), PKCε (SC-1681) (1:500; Santa Cruz). Membranes were stripped and re-probed with Caspase 3 and GRIP1 antibodies to serve as the loading controls for cytosolic and membranous fractions.

Electrophysiological Studies

To elucidate the impact of prenatal cocaine exposure on AMPAR function during brain maturation, electrophysiological recording that focuses on AMPAR function was performed in 400 μm thick medial prefrontal cortical slices from P21, P40-, P60- and P180 prenatally cocaine- or saline-exposed (and some treatment naïve) rats. This procedure was performed as described previously (Jia et al., 1996), with modifications so as to determine AMPAR-mediated LTD and LTP.

Brain slice preparation: Briefly, the rat is anesthetized (60 mg/kg pentobarbital sodium) and the cranium is opened quickly. The brain is removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) saturated with 5% CO_2 and 95% O_2 for 2 minutes, then blocked and sectioned into 400 μm coronal slices using a Vibratome. Slices are incubated at 25°C in well-aerated ACSF for 2 hr, and then placed in a submersion-type recording chamber (Warner Instr.) superfused with aerated ACSF at 25°C.

Electrophysiologic recording: Evoked field potential in layer III of rat prefrontal cortical slices were recorded using a conventional method. A glass micropipette (with 5 μm tip) filled with 1M Na acetate was placed on the prelimbic area 300 μm from pia. Single electrical pulses (0.1 ms, 0.05Hz, about 2x threshold strength) were delivered through a concentric bipolar electrode (FHC) placed on the medial edge (i.e. layer I/II) and 100μm lateral to the radial axis of the recording micropipette to favor activation of axons from near-by columns (i.e. off-beam stimulation). Voltage responses were led to a d.c amplifier (Axoclamp 2a), then digitized by a 16 bit analog-to-digital board (Measurement computing Corp.) operated through data-acquisition software (Snapmaster 3.5). The responses were averaged over 4 frames to improve signal-to-noise ratio, then further

processed in real-time and subsequently. Neurochemical reagents such as picrotoxin were applied through a side-tube in the superfusion line.

LTD was elicited by a single train of low frequency stimulation (2.4 Hz of triplets at 500Hz, 10 min). After the train, responses evoked at the same intensity as in baseline condition were recorded and plotted against time to reveal the persistent depression compared with the baseline.

Immunoaffinity purification of native GRASP-1

To isolate native GRASP-1, frontal cortices of P21 prenatally cocaine- or saline-exposed rat brains were homogenized in hypo-tonic homogenization solution described above. 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 20 min at 25ºC followed by 60 min at 4ºC with end-over-end constant shaking. Following centrifugation to remove insoluble debris, the obtained brain lysate was diluted 5-fold and GRASP-1 was individually purified using immunoaffinity column (Seize-X immunoprecipitatation kit, PIERCE) with covalently immobilized antibodies directed against GRASP-1 according to the manufacturer's instruction. GRASP-1 was eluted twice with 90 µl antigen elution buffer (PIERCE). The resultant elutes were then neutralized immediately with 20 µl 1.5M Tris HCl, pH8.8 and concentrated to 100 µl passing through 10-KDa cut-off filter (Cole-Palmer). The protein concentration was determined using the Bradford method (Bio-Rad).

***In vitro* determination of GRASP-1 – GRIP1 interaction and immunoprecipitation**

To control GRIP1 phosphorylation state, native GRIP1 proteins (5 μg) purified from frontal cortices of 21-day-old prenatally saline- and cocaine-exposed rats were incubated with 100 μg/ml alkaline phosphatase (Sigma) in Tris, pH8.0, 130 mM NaCl, protease and phosphatase inhibitors at 30ºC for 20 min. The phosphatase activity was terminated by 10 mM NaF/ 1 mM Na_3VO_4 and specific PKC- and src-mediated phosphorylation was induced by incubation with 0.5 μg/ml recombinant γPKC (Calbiochem), 20 μg phosphotidylserine or recombinant Src in the presence of 30 μM ATP in Kreb's-Ringer at 30ºC for 10 min. Purified GRASP-1 (5 μg) protein from gestational saline- and cocaine-exposed rat brain was individually added and incubated in 100 μg/ml brain phospholipids, 1% bovine serum albumin (BSA)-containing Kreb's-Ringer at 30°C for 30 min with constant shaking. The GRIP1-associated GRASP-1 was then isolated along with GRIP1 by 20 μl immobilized anti-GRIP1 conjugated protein A-agarose beads. The immunoprecipitates were size-fractionated on 7.5% SDS-PAGE and the levels of GRASP-1 were determined by Western blot analysis using specific antibodies. The obtained blots were screened for phosphoserine first using anti-phosphoserine, stripped and re-probed twice sequentially with anti-phosphotyrosine and anti -GRIP1. The signals were detected using a chemiluminescent method (Pierce) and visualized by exposing to x-ray film.

GDP Dissociation Assay to assess GRASP-1 rasGEF activity

GRASP-1 rasGEF activity in the FCX of P21 *in utero* cocaine- and saline-exposed rats was measured using purified GRASP-1, by a method described by Ye et al.

(2001) with some modifications. To load ras with [^{3}H]GDP, 0.5µg H-ras (Upstate Biotechnology/Chemicon) was incubated with 10µCi [^{3}H]GDP in 50µl nucleotide loading buffer (50mM Tris with 10mM EDTA, 5mM $MgCl_2$, 1mM DTT, and 1mg/ml BSA) at 37^0 C. Twenty minutes later 60µl of nucleotide loading stopping buffer (50mM Tris-HCL [pH7.4], with 5mM $MgCl_2$, 1mM DTT and 1 mg/ml BSA) was added. The 110µl sample was divided into two, and each sample was added to 500µl dissociation reaction buffer (25mM Tris-HCL [pH 7.4] with 2 mM $MgCl_2$, 1mM DTT, 1 mg/ml BSA, and 0.1mM GDP) containing 5µg of purified GRASP-1 from frontal cortical synaptosomes of prenatally cocaine- and saline-treated rats, respectively. The two reactions were incubated at 37^0 C. A sample was then removed from each mixture at 1, 2, 4, 8 and 20 min time points and mixed with 200µl ice-cold dissociation reaction stopping buffer (50mM Tris-HCL [pH 7.4] with 10mM $MgCl_2$). Rapid filteration was performed using GF/C filters under vacuum. The filter was washed twice with an ice-cold stopping buffer (5 ml each), and the radioactivity was measured by liquid scintillation counting after air dry. The data were expressed as the percent of [^{3}H]GDP bound at each time point comparing to input [^{3}H]GDP level (at time 0).

Affinity precipitation of GTP RhoA, Rap1 and Rac1/Cdc42

GTP bound RhoA, Rap1 and Rac-1/Cdc42 were affinity purified from the synaptosomal enriched fraction (method described above) of the FCX region from prenatal cocaine- and saline- treated rats. The precipitation of active Rho was performed using the fusion protein GST-Rhotekin, which specifically recognizes the active GTP-bound form of RhoA. Similarly, GTP-Rap1 was precipitated using fusion protein GST-RalGDS and Rac1/Cdc42 from GST-Pak1 respectively. The synaptosomes were ruptured

by sonicating in 0.25 ml of immunoprecipitation buffer and solubilized using 0.5% digitonin, 0.2% sodium cholate and 0.5% NP-50 at 4°C for 1 hr. Following dilution with 0.75 ml of immunoprecipitation buffer and centrifugation, the GTP-bound RhoA, Rap1 and cdc42/Rac1 in the resultant synaptosomal lysates were purified by incubating at 4^{o} C for 1 hour with Rhotekein-RBD beads (10 μg) (Upstate Technologies), GST-RalGDS (10 μg) (Upstate Technologies) and GST-Pak beads (10 μg) (Upstate Technologies), respectively with end-over-end rotation. Following centrifugation, the beads were washed twice with 1 ml of ice-cold 25 mM Tris HCl, pH 7.4, 20 mM $MgCl_2$ containing protease and protein phosphatase inhibitors. To determine the levels of GTP-RhoA, GTP-Rac1/Cdc42, and GTP-Rap-1 the proteins were size fractionated on 12% SDS-PAGE and Western blot analysis was performed using antibodies specific to RhoA, Rac-1/Cdc42, and Rap1 (Santa Cruz). The signals were detected using a chemiluminescent method (Pierce) and visualized by exposing to x-ray film.

Determination of F-actin levels

To measure the level of F-actin, synaptosomes were prepared from the FCX of prenatally cocaine- and saline-treated P21 rats by method described above. The synaptsomes (500 μg) were treated with vehicle, alkaline phosphatases (1 mg/ml) or alkaline phosphatases (1 mg/ml) plus phosphatase inhibitors (10 mM NaF/1 mM Na_3VO_4) or cytochalasin D (10 μM) *in vitro* for 20 min at 30^{o} C in 50 mM Tris HCl, pH8.0, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, protease inhibitors (total incubation volume: 100 μl). Following termination of reaction by diluting with 300 μl immunoprecipitation buffer, the synaptosomes were solubilized in 0.5% digitonin, 0.2% sodium cholate, and 0.5% NP-40 and Actin-containing proteins were extracted by

incubating with 2 μg of biotin-conjugated anti-actin antibodies (Santa Cruz) for 1 hr at 4°C and the anti-actin linked actin proteins were then immobilized by loading 50% of reaction mixture into each well of the streptavidin-coated 96-well plate (Pierce) and incubating at 4°C for 1 hr. The solution was then removed and the plate was washed twice with 25 mM Tris HCl, pH 7.4; 100 mM NaCl (200 μl/well). The level of F-actin was then determined using rhodamine-conjugated phalloidin (0.5 μl/well) (Molecular Probes/Invitrogen). After two washes with 100 μl 25 mM Tris, [pH7.4] containing 100 mM NaCl, the fluorescence intensity of phalloidin was measured using Beckman multimode plate reader, DX880.

Data Analysis and Statistical Evaluation

Statistical differences between cocaine and saline groups were assessed using the two-tailed Student's t test. Differences between *in vitro* dose-response relations were analyzed by ANOVA followed by Newman Keul's test for multiple comparisons.

CHAPTER-3

EXPERIMENTAL RESULTS

Prenatal cocaine exposure reduces synaptic membrane expression of GluR1 subunit in the FCX

To assess the influence of prenatal cocaine exposure on the expression of AMPAR subunits, pregnant rats were subcutaneously injected with cocaine and saline throughout 21 days of gestation. The total levels of GluR1, GluR2 and GluR3 subunits in the frontal cortex (FCX) of the prenatal cocaine and saline exposed pups were examined at postnatal day 21 (P21) using western blot analysis. Because the synaptic membrane recruitment of AMPAR subunits determines the strength of basal synaptic transmission and synaptic plasticity, we also studied the effect of prenatal cocaine exposure on the membrane recruitment of various AMPAR subunits. In pilot experiments, the synaptic membrane expression of AMPAR subunit GluR1 was determined in frontal cortices of 21-days old prenatally cocaine- and saline-exposed rats. The synaptic levels of AMAPR subunit GluR1 was measured in synaptosomes isolated from frontal cortices by western blotting the with specific anti-GluR1 antibody. The western blot shows a drastic reduction in the membrane-associated GluR1 in prenatal cocaine-exposed brains compared with the saline treated controls (figure 3.1). Since the synaptic recruitment of GluR1 containing AMPARs plays a crucial role in activity-dependent synaptic potentiation, a reduced membrane-associated GluR1 is indicative of deficient AMPAR mediated synaptic transmission in the prenatal cocaine exposed brain.

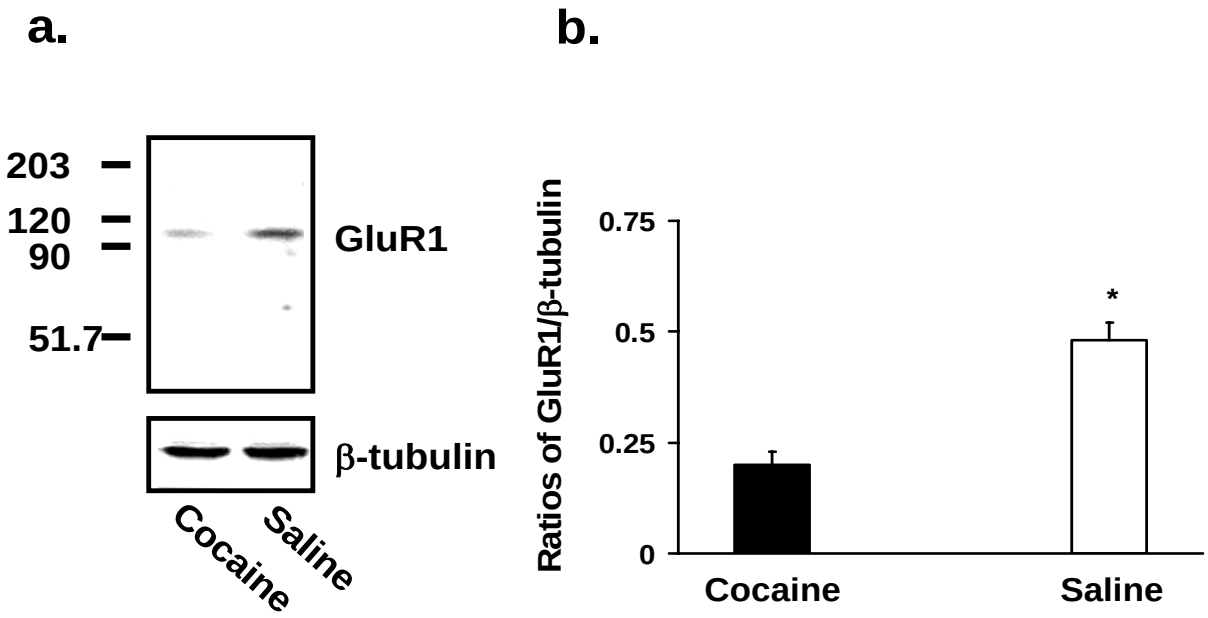

Figure. 3.1. Synaptic membrane expression of GluR1 is markedly reduced in the frontal cortex of prenatal cocaine-exposed rats. (**a**)The particulate fractions of the synaptosomes were prepared from frontal cortices of 21-day-old rats that have had exposure to saline or cocaine during gestation. Following solubilization by boiling in SDS-containing sample preparation buffer, 50 μg solubilized membranous proteins were size-fractionated on 7.5% SDS-PAGE and Western blots were performed with anti-GluR1 (1:500) antibody. The blots were stripped and re-probed with anti-β-tubulin to confirm equal loading. The blot shown is the representative of 4 individual experiments each used frontal cortices from one rat from each treatment group. **(b)** Densitometric quantification of blots shown in *A*. $n = 4$. Data are means ± s.e.m. of the ratios of optical intensity for GluR1 to that of β-tubulin. * $p < 0.01$ comparing the GluR1/β-tubulin ratios in prenatal saline- and cocaine-exposed rats by two-tailed Student's *t*-test.

Prenatal cocaine exposure reduces the synaptic membrane expression of GluR2 and GluR3 subunits without affecting the levels of PDZ domain binding partners in FCX of P21 rats

The GluR2 and GluR3 subunits form the constitutive cycling pool of AMPARs that continuously cycle between the postsynaptic membrane and intracellular compartments and are responsible for maintaining basal synaptic activity and LTD expression (Passafaro et al., 2001; Shi et al., 2001). Therefore, in the first set of experiments, we measured the total level as well as the levels of AMPAR subunits GluR2 and GluR3 in both the cytosol and postsynaptic membranes prepared from prenatal saline- and cocaine-exposed P21 rat brains. First, we measured the expression level of GluR2 and GluR3 in the synaptosomal fraction prepared from the frontal cortices of prenatal cocaine and saline exposed brain. The western blot shows similar levels of GluR2 and GluR3 proteins in prenatal cocaine and saline exposed FCX (figure 3.2). In the next sets of experiments we measured the level of GluR2 and GluR3 in the cytosol and synaptic membranes of synaptosomes prepared from the frontal cortices of prenatal cocaine and saline exposed brain. The western blot shows that the majority of GluR2 (89.7 ± 6.2%) and GluR3 (94.1 ± 5.1%) are located in synaptic membranes of saline-treated brains (figure 3.3). However, there is a dramatic increase in the cytosolic levels of GluR2 (62.3 ± 5.1%) and GluR3 (58.6 ± 4.5%) in the prenatal cocaine-exposed brains (figure 3.3). These data show that in the prenatal cocaine-exposed brain majority of the AMPAR subunits are located in the cytosol, whereas in the saline treated rats the subunits are localized to the synaptic membrane.

Since the constitutive cycling pool of GluR2-, and GluR3-containing AMPARs play a crucial role in the maintenance of basal synaptic transmission and LTD expression (Kim et al., 2001; Chung et al., 2000), the next sets of experiments are designed to test whether AMPAR-regulated field potential and LTD are reduced in brain slices derived from prenatal cocaine-exposed rats. The magnitude of AMAPR-mediated LTD was measured in the presence of $GABA_AR$ blockade by picrotoxin in the medial prefrontal cortical slices (mPFC) of 25-day-old prenatal saline- and cocaine-exposed rats. The AMPAR components were validated by their sensitivity to specific AMPAR antagonist, CNQX. The data in figure 3.4A shows the presence of CNQX-sensitive AMPAR-mediated field potential response. In response to a train of low-frequency stimulation, the sustained depression of synaptic potential response (LTD) is evidenced at both 6- and 18-min time points in control rats (figure 3.4B) - but dramatically reduced in prenatal cocaine-exposed rats (figure 3.4C). These results suggest a functional correlate of the reduced synaptic membrane recruitment of GluR2 and GluR3 in the prenatal cocaine-exposed brain shown in figure 3.3.

Together with unchanged total protein expression of AMPAR subunits in prenatal cocaine- and saline-exposed brains (figure 3.2), the reduced synaptic membrane levels of GluR2 and GluR3 concurrent with higher cytosolic levels of the proteins (Figure 3.3) suggest that prenatal cocaine exposure attenuates the synaptic membrane targeting of GluR2 and GluR3. It is known that the synaptic membrane expression of GluR2 and GluR3 containing AMPARs is modulated by their phosphorylation state and interaction with the PDZ domain containing synaptic scaffolding proteins. Therefore, we first considered changes in the $pSer^{880}$-GluR2 and $pSer^{891}$-GluR3 levels, since PKC-mediated

phosphorylation of GluR2/3, promotes their interaction with PDZ-domain containing protein PICK and subsequent internalization (Chung et al., 2000). We examined whether prenatal cocaine exposure leads to changes in pSer^{880}-GluR2 and pSer^{891}-GluR3 levels as well as the expression levels of PDZ-domain containing GluR2/3 interacting proteins, GRIP1, GRIP2, PICK, ABP and a non-PDZ protein NSF. Western blot analyses show no discernible changes in pSer^{880}-GluR2, pSer^{891}-GluR3 (figure 3.5) in prenatal cocaine- and saline-exposed brain. Moreover, the levels of AMPAR associated PDZ-domain containing proteins GRIP1, GRIP2, PICK, ABP, and no PDZ protein NSF (Figure 3.6 and 3.7) remain similar in the prenatal cocaine- and saline-exposed rats. Collectively, the data demonstrate that prenatal cocaine exposure reduces synaptic recruitment of GluR2 and GluR3 subunits.

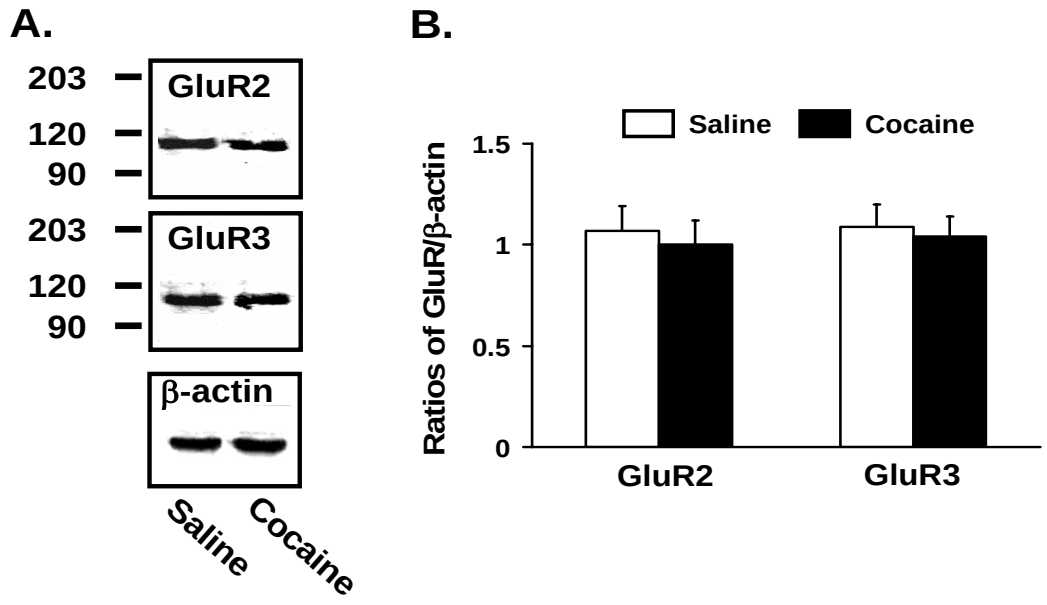

Figure. 3.2: Prenatal cocaine exposure did not alter the expression levels of GluR2 and GluR3 in the FCX. (A) The expression level of GluR2 and GluR3 in post-mitochondrial synaptosome-enriched fraction prepared frontal cortices of 21-days-old rats exposed to saline or cocaine *in utero* were compared by Western blotting. The proteins were size-fractionated on 7.5% SDS-PAGE and Western blots were performed with anti- GluR2 and -GluR3 (1:500). The blots were stripped and sequentially re-probed with β-actin to validate equal loading. The blot represents *n* =4 rats from each treatment group. (B) Densitometric quantification of the western blot. Data are means ± s.e.m. of the ratio of GluR2/3 to β-actin. There were no statistical differences between prenatal saline- and cocaine-exposed rats in these measures.

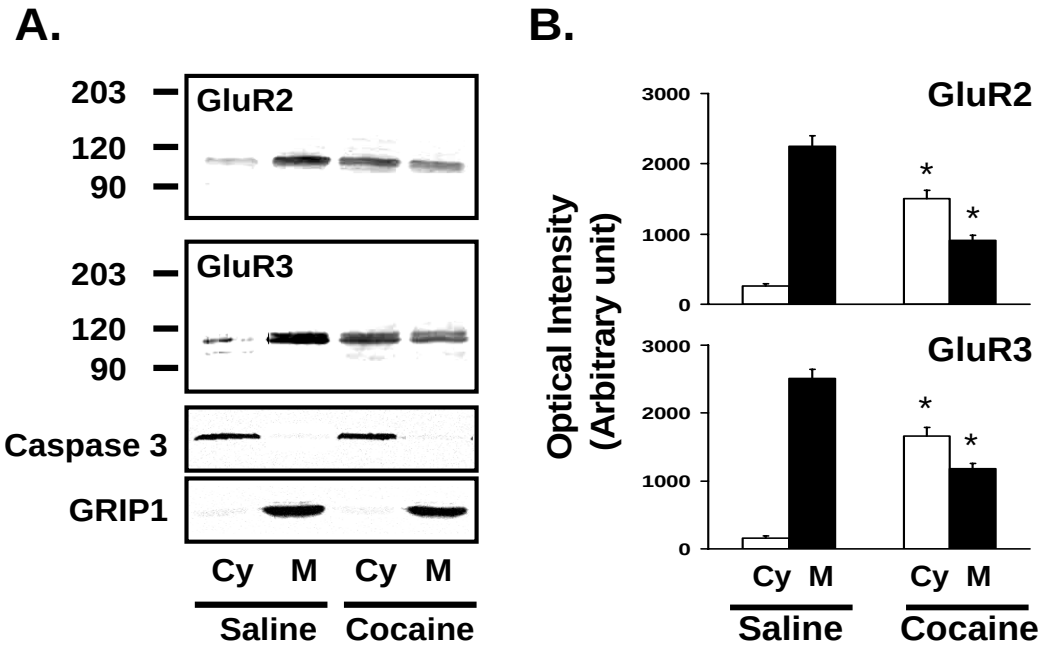

Figure. 3.3: Cellular distribution of GluR2 and GluR3. The synaptic expression of GluR2/3 is markedly reduced in the frontal cortex (FCX) of prenatal cocaine-exposed rats. **(A)** Cytosolic (Cy) and membranous (M) fractions of the synaptosomes were prepared from FCX of 21-day-old rats exposed to saline or cocaine during gestation. Following solubilization by boiling in SDS-containing sample preparation buffer, 50 μg solubilized proteins were size-fractionated on 7.5% SDS-PAGE and Western blots were performed with anti- GluR2 and GluR3 (1:500) antibody. The blots were stripped and sequentially re-probed with anti-caspase-3 (1187 ± 107 and 1210 ± 99 optical intensity in saline and cocaine, respectively) and GRIP1 **(2509** ± 132 and 2319 ± 146 optical intensity in saline and cocaine, respectively) to validate equal loading in cytosolic and membranous extracts. The blot is a representative of 4 individual rats from each treatment group. **(B)** Densitometric quantification of blots. $n = 4$. Data are means ± s.e.m. of the optical intensity. * $p < 0.01$ comparing the level of receptor in respective cytosolic and membrane fractions.

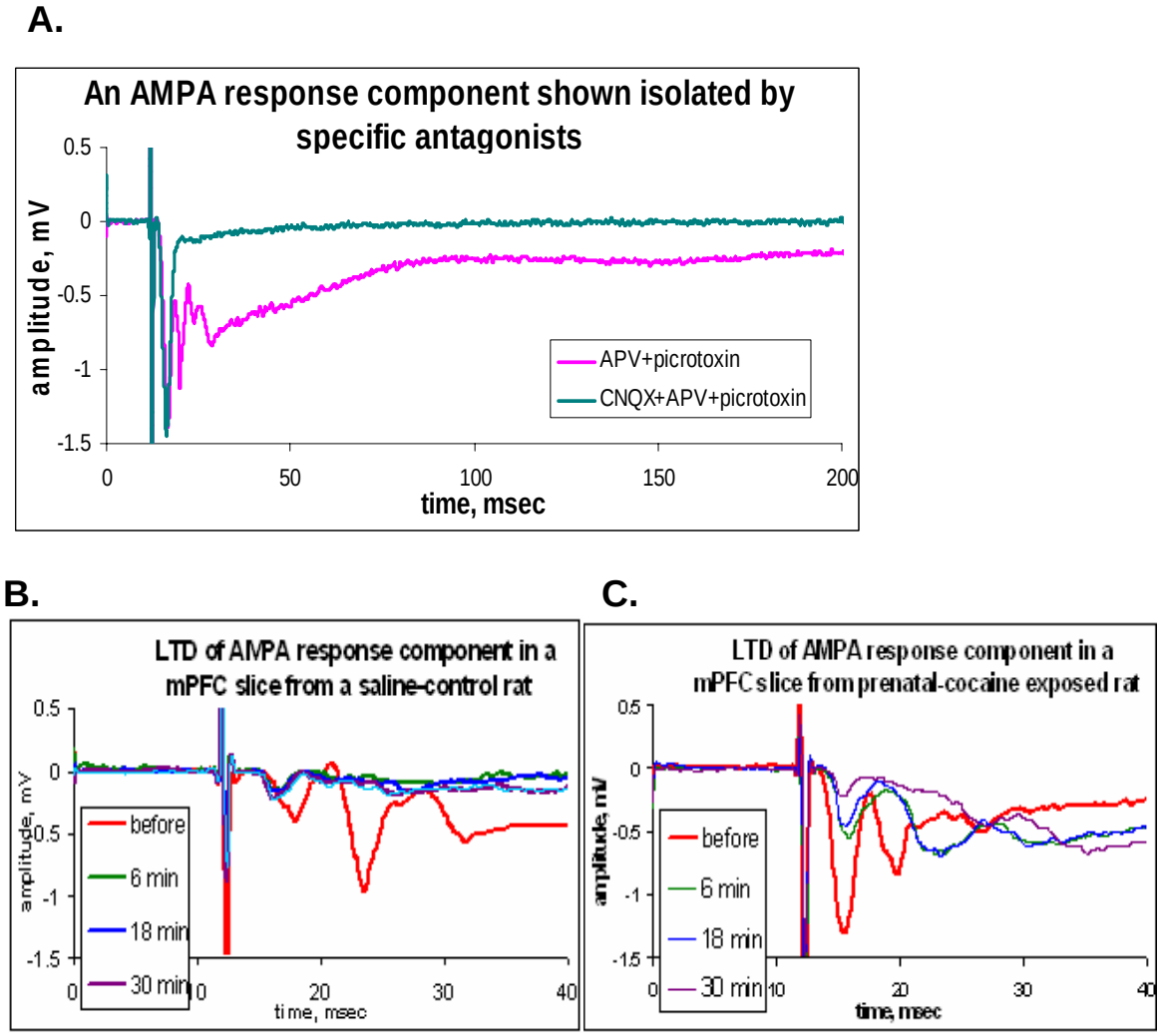

Figure. 3.4: Reduced AMPAR mediated LTD in the mPFC slices from prenatally cocaine exposed rats. LTD was elicited by a single train of low frequency stimulation (triplets of 2.4 Hz at 500Hz, 10 min). After the train, responses evoked at the same intensity as in baseline condition were recorded and plotted against time to reveal the persistent depression compared with baseline. (A) Shows the detection of CNQX-sensitive AMPAR-mediated field potential response. In response to a train of low-frequency stimulation, a sustained depression of synaptic potential response (LTD) is evidenced in both 6- and 18-min time points in prenatally saline (B)- but dramtically reduced in prenatal cocaine-exposed rats (C).

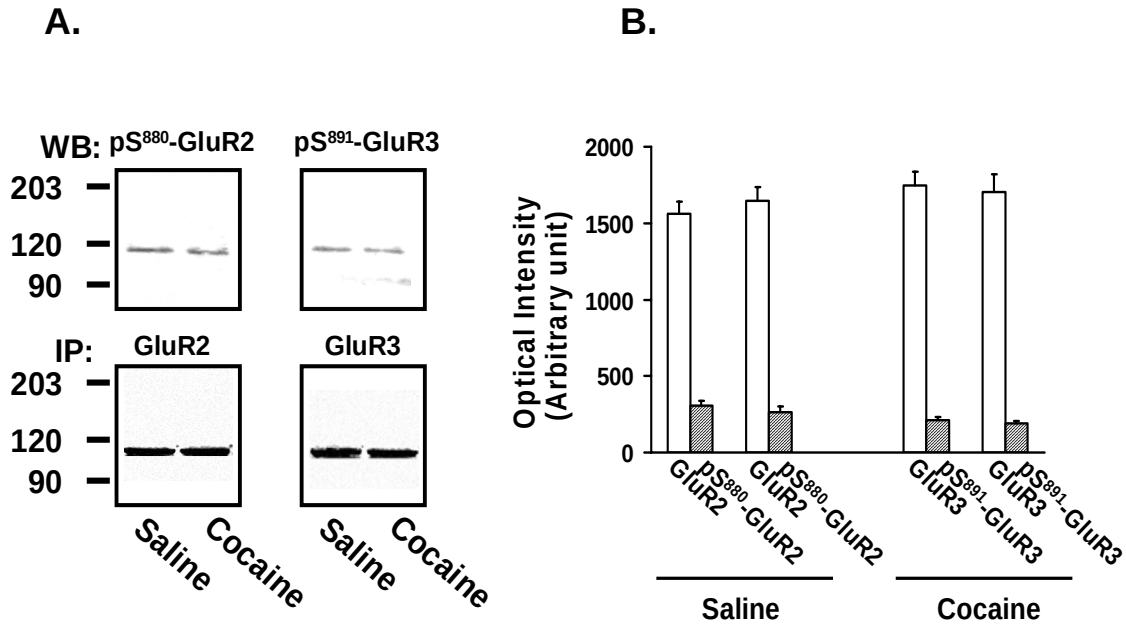

Figure. 3.5: Prenatal cocaine exposure did not alter the expression levels of pSer^{880}-GluR2 and pSer^{891}- GluR3 (A) The expression levels of pSer^{880}-GluR2 and pS^{891}-GluR3 in post-mitochondrial synaptosome-enriched faction prepared from frontal cortices of 21-day-old rats exposed to saline or cocaine *in utero* were compared by Western blotting following immunoprecipitation with anti-GluR2 or -GluR3. Following solubilization of anti-GluR2 and -GluR3 immunoprecipitates by boiling in SDS-containing sample preparation buffer, immunoprecipitates derived from 50 μg of brain lysate were size-fractionated on 7.5% SDS-PAGE and Western blots were performed with anti- pSer^{880}-GluR2 and -pS^{891}-GluR3 (1:500). The blots were stripped and sequentially re-probed with anti-GluR2 and -GluR3 to validate immunoprecipitation efficiency. The blot is a representative of 4 individual rats from each treatment group. (B) Densitometric quantification of the blot $n = 4$. Data are means ± s.e.m. of the optical intensity. There were no statistical differences between prenatal saline- and cocaine-exposed rats in these measures.

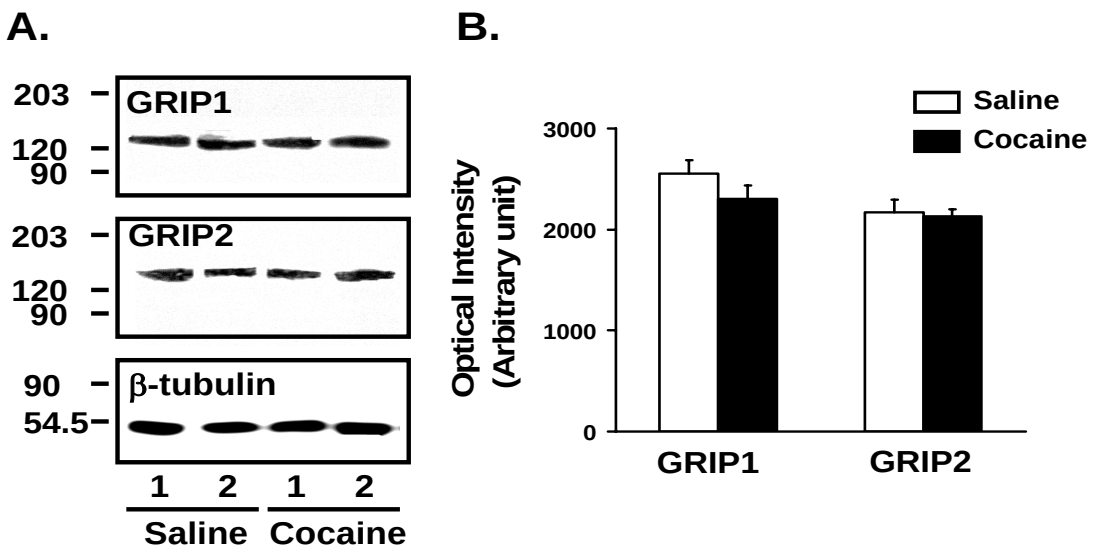

Figure. 3.6: Prenatal cocaine exposure did not alter the expression levels of GRIP1 or GRIP2. (A) The expression levels of GRIP1 and GRIP2 in post-mitochondrial synaptosome-enriched faction prepared from frontal cortices of 21-day-old rats exposed to saline or cocaine *in utero* were compared by Western blotting. Following solubilization of the brain lysate by boiling in SDS-containing sample preparation buffer, 50 μg solubilized brain lysate was size-fractionated on 7.5% SDS-PAGE and Western blots were performed with anti-GRIP1 and –GRIP2 (1:500). The blots were stripped and sequentially re-probed with anti-β-tubulin to validate equal loading. The blot is a representative of 4 individual rats from each treatment group. (B) Densitometric quantification of the blot $n = 4$. Data are means ± s.e.m. of the ratios of the indicated protein to loading control. There were no statistical differences between prenatal saline- and cocaine-exposed rats in these measures.

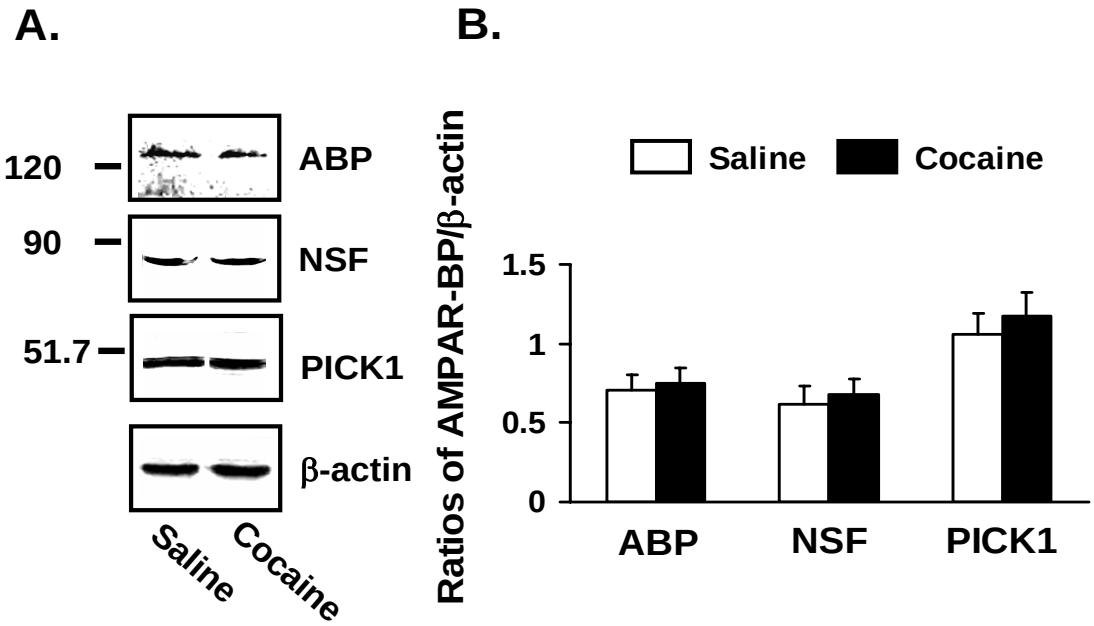

Figure. 3.7: Total levels of AMPAR GluR2 and GluR3 binding proteins (BP) ABP, NSF, and PICK1. Prenatal cocaine exposure had no effect on AMPAR binding proteins, NSF and PICK1 in the frontal cortex. (**a**) The post-mitochondrial fractions were prepared from frontal cortices of 21-day-old rats that have had exposed to saline or cocaine during gestation. Following solubilization by boiling in SDS-containing sample preparation buffer, 50 μg solubilized lysates were size-fractionated on 10% SDS-PAGE and Western blots were first performed with anti-ABP (1:1000). The blots were stripped and sequentially re-probed with anti-NSF (1:500), -PICK1 (1:500) and finally -β-actin to confirm equal loading. The blot shown is the representative of 4 individual experiments each used frontal cortices from one rat from each treatment group. (**b)** Densitometric quantification of blots shown in *A*. $n = 4$. Data are means ± s.e.m. of the ratios of optical intensity for AMPAR-BP to that of β-actin. There are no discernible changes in the levels of ABP, NSF, PICK1 and β-actin in frontal cortices from prenatally saline- and cocaine-exposed (two-tailed Student's *t*-test)

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Reduced association between GRIP1/2 and GluR2/3 in the frontal cortex of prenatal cocaine exposed rats *in vitro*

Numerous studies have established that the interaction of AMPARs with the PDZ domain proteins GRIP1/2 regulates the synaptic membrane localization of GluR2 and GluR3. Because we observed higher cytosolic levels concurrent with reduced synaptic membrane levels of GluR2 and GluR3 (figure 3.2) subunits in the prenatal cocaine-exposed rats without any changes in the membrane levels of GRIP1/2 (figure 3.5), the next set of experiments were designed to directly test whether there were any changes in the interaction between GluR2/3 and its PDZ domain binding partner GRIP1. The impact of prenatal cocaine exposure on the interaction between GluR2/3 and its scaffolding protein GRIP was measured *in vitro*. In these experiments, GluR2, GluR3 and GRIP1 in their native form were individually purified from the FCX of prenatal cocaine and saline treated rats using immunoaffinity columns with immobilized specific antibodies directed against each of the proteins of interest. A single protein band with identical molecular weight of the target protein for each affinity-purified protein was observed using Western blot analysis (data not shown). The magnitude of interaction between purified GluR2/GluR3 and GRIP1 from either prenatal cocaine- or saline-exposed rats was assessed *in vitro* by incubating in Kreb's Ringer containing brain phospholipids and bovine serum albumin (BSA). After capturing the GRIP1-associated GluR2 and GluR3 by co-immunoprecipitation with anti-GRIP1, GluR2 or GluR3 levels were then determined using western blotting. The data showed in figure 3.8 indicate that GRIP1 from saline-treated rats interacts equally well with GluR2 and GluR3 from both prenatal cocaine- and saline-exposed brains. In contrast, there was a significant reduction in

association between GRIP1 from prenatal cocaine-exposed rats and GluR2/GluR3 regardless of the source of the receptors (figure 3.8). These data suggest that the reduced synaptic membrane targeting of GluR2/3 in the prenatal cocaine-exposed brain is caused by alteration made on GRIP1 but not the receptors. The fact that GluR2/3 interaction with PICK remains similar (figure 3.9), these data suggests that prenatal cocaine exposure uniquely modifies GRIPs through which a reduction in GluR2 and GluR3 – GRIP interaction is observed.

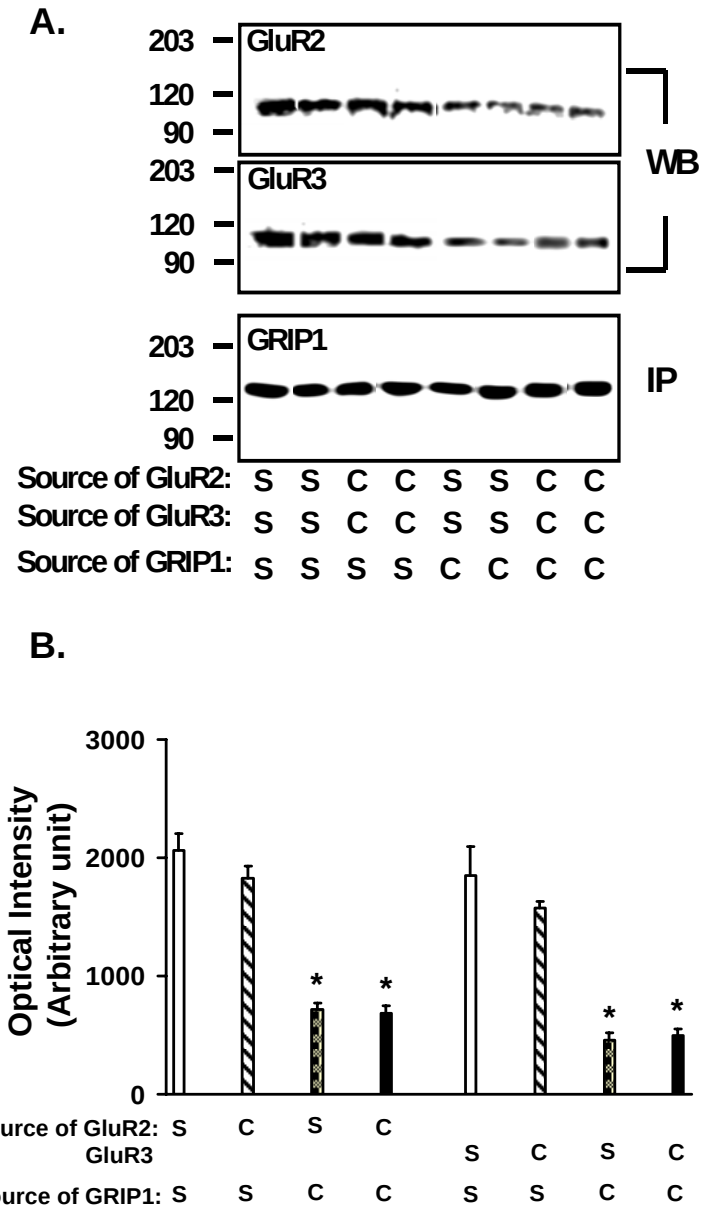

Figure. 3.8: Reduced association between GluR2/GluR3 and GRIP1 in the frontal cortex of prenatal cocaine exposed rats. (A) Native GRIP1, GluR2 and GluR3 were individually purified from frontal cortices of 21-days-old rats exposed to saline (**S**) or cocaine (**C**) during gestation by immunoaffinity columns with immobilized antibodies in the presence of protease and protein phosphophatase inhibitors. The interaction between GluR2/3 with GRIP1 was assessed *in vitro* by incubating 5 μg of each protein in 100 μg/ml brain phospholipids, 1% BSA-containing Kreb's-Ringer at 30°C for 30 min with constant shaking. The GluR2- or GluR3-GRIP1 complexes were isolated along with GRIP1 by immunoprecipitation with anti-GRIP1 and the levels of GluR2 or GluR3 associated with GRIP1 were determined by Western blotting. The blot is a representative of 4 separate experiments each using a protein preparation from one rat in of the two groups. (B) Densitometric quantification of blots. $n = 4$. Data are means ± s.e.m. of optical intensities. * $p < 0.01$ comparing the level of GluR2 or GluR3 with the group that

GRIP1 and receptor are both isolated from prenatal saline-treated rats by Newman-Keuls test for multiple comparisons that followed one-way ANOVA.

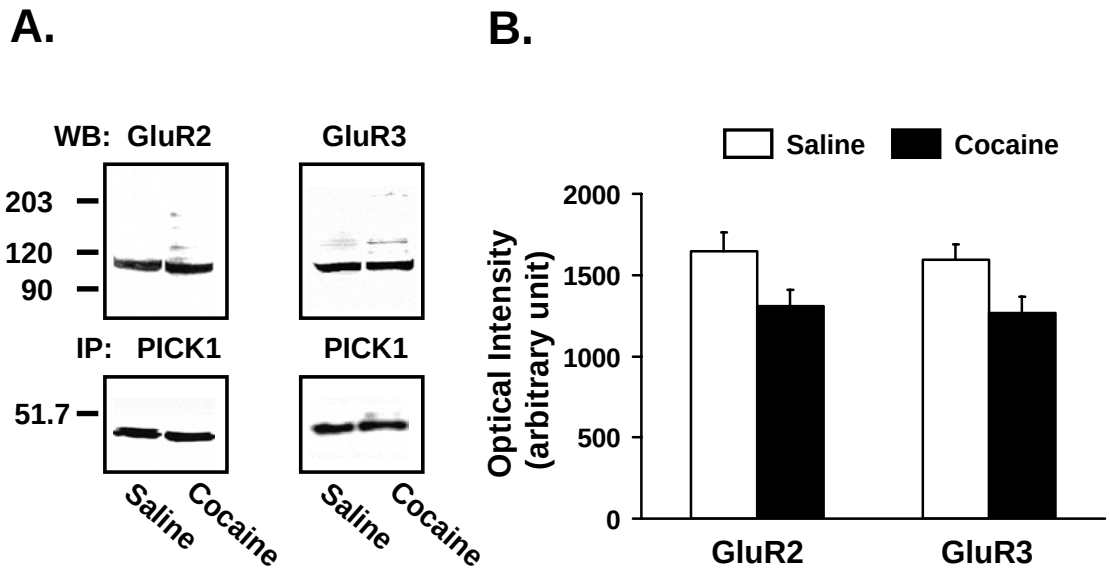

Figure. 3.9: Prenatal cocaine exposure has no effect on association between GluR2/GluR3 and PICK1 in the frontal cortex. (A) PICK1, GluR2 and GluR3 were individually purified from frontal cortices of 21-day-old rats that have had exposure to saline or cocaine during gestation by immunoaffinity columns with immobilized antibodies in the presence of protease and protein phosphophatase inhibitors. The interaction between GluR2 and GluR3 with GRIP1 was assessed *in vitro* by incubating 5 μg of each protein in 100 μg/ml brain phospholipids, 1% BSA-containing Kreb's-Ringer at 30°C for 30 min with constant shaking. The GluR2- or GluR3-PICK1 complexes were isolated along with PICK1 by immunoprecipitation with anti-PICK1 and the levels of GluR2 or GluR3 associated with PICK1 were determined by western blotting. The blot shown is representative of 4 separate experiments each used a protein preparation from one individual rat in prenatally saline- or cocaine-treated groups. (**b**) Densitometric quantification of the blots. $n = 4$. Data are means ± s.e.m. of the optical intensity. There are no detectable differences in the levels of PICK1 - GluR2 or - GluR3 association between prenatally saline- and cocaine-treated rats (one-way ANOVA).

Serine and Tyrosine phosphorylation of GRIP1 and GRIP2 are increased in frontal cortices of prenatal cocaine exposed animals

PKC mediated phosphorylation of GluR2 at Ser-880 and GluR3 at Ser-891 residues have been shown to increase their interaction with PICK leading to their internalization (Chung et al., 2000). Our results show no changes in expression levels of Ser^{880}-GluR2 or Ser^{891}-GluR3 (figure 3.4), PICK (figure 3.6), and similar magnitude of GluR2-, GluR3- and PICK interaction (figure 3.9) in the synaptic membrane of the FCX of prenatal saline- and cocaine-exposed rats. These data suggest that the reduced synaptic membrane localization of GluR2 and GluR3 in FCX of prenatal cocaine-exposed rats is not caused by a heightened PICK-GluR2/3 interaction due to increased PKC-phosphorylation on GluR2/3. We then consider the possibility that the blockade of synaptic membrane expression of GluR2 and GluR3 originates from changes made in GluR2 and GluR3 synaptic interacting protein, GRIP. Among many probable mechanisms, we first investigated putative phosphorylation sites on GRIP1 and GRIP2 since they contain numerous putative PKC phospohorylation sites. Moreover, we examined the effects of phosphorylation of GRIP on GluR2/3-GRIP interaction. In these experiments, immunopurified native GRIP1 and GRIP2 from the FCX of prenatal cocaine- and saline-treated rats were immunoblotted with antibodies against phospho-serine, -threonine and -tyrosine. We found a marked increase (2-2.5 fold) in phosphorylation of GRIP1 on serine and tyrosine residues (97 ± 11 % and 151 ± 9 %, respectively) in the prenatal cocaine-exposed FCX (figure 3.10). These data indicate that a persistent GRIP1 and GRIP2 phosphorylation on serine and tyrosine occurs in FCX of prenatal cocaine-exposed rats and suggest that the heightened GRIP1/2 phosphorylation

may be relevant to reduction in GRIP – GluR2/3 interaction following cocaine exposure during gestation.

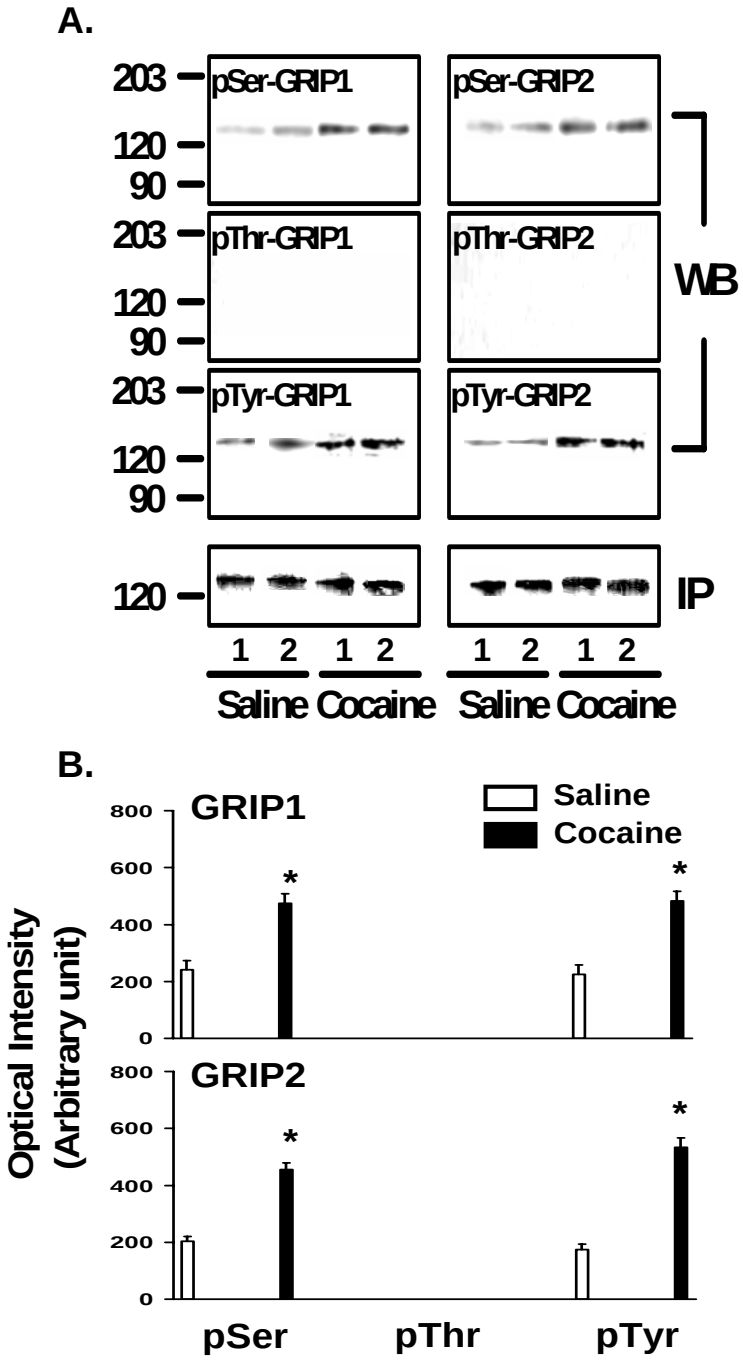

Figure. 3.10: Serine (Ser), Threonine (Thr) and Tyrosine (Tyr) phosphorylation of GRIP1. Enhanced serine and tyrosine phosphorylation of the GRIP1 in frontal cortices of prenatal cocaine-exposed rats. (A) Native GRIP1 and GRIP2 proteins were isolated from frontal cortical lysate derived from prenatal saline- and cocaine-exposed 21-days-old rats by immunoprecipitation with anti-GRIP1 antibody. The levels of Serine (**Ser**), Threonine (**Thr**) and tyrosine (**Tyr**) phosphorylation on the purified GRIP1 or GRIP2 derived from 50 μg of frontal cortical lysate was determined by Western blot using specific antibodies directed against each phospho-epitope. The blots are representatives of 4 individual preparations each using one rat from each group. (B) Densitometric

quantification of blots. $n = 4$. Data are means ± s.e.m. of the optical intensity. $*p < 0.01$ comparing the levels of phosphorylated GRIP1 and GRIP2 isolated from prenatal saline-treated rats by two-tailed Student's *t* test. There was no significant difference in the level of GRIP1 immunoprecipitated from prenatal saline- and cocaine-treated rats.

PKC- and Src-mediated GRIP1 phosphorylation is responsible for the attenuated coupling of GRIP1 to GluR2/3

Although GRIPs possess several putative phosphorylation sites for PKC and other kinases, the effect of altered GRIP phosphorylation states on GRIP-GluR2 interaction is currently not clear. Our findings show that an increase in serine and tyrosine phosphorylation of GRIP (Figure 3.10) lead us to hypothesize that a persistent increase in phosphorylation of discrete sites on GRIPs found in brains of prenatal cocaine-exposed rats may be involved in a reduced GluR2/3-GRIP interaction and hence by dephosphorylating GRIP this interaction may be restored. To explore this possibility, the phosphorylation state of purified GRIP1 from the FCX of prenatal cocaine- and saline-exposed rats was manipulated using alkaline phosphatase and recombinant protein kinases and the interaction between GRIP1 and GluR2 was determined in an *in vitro* setting. In figure 3.11, we show that preserving the native phosphorylation state of the purified GRIP with protein phosphatase inhibitors, sodium fluoride and sodium vanadate which inhibits serine/threonine and tyrosine protein phosphatases, significantly reduces GluR2 – GRIP1 interaction in the prenatal cocaine-exposed brain (figure 3.11 Lane 1 & 2). This prenatal cocaine-mediated effect was abolished by dephosphorylation of the GRIP1 with alkaline phosphatases (1 mg/ml) as this treatment restores the interaction between GRIP1 and GluR2 to control levels (figure 3.11 lane 3 & 4). More importantly, incubation of dephosphorylated GRIP protein with recombinant γPKC (Figure 3.11 lanes 5 & 6) and Src in the presence of ATP, essential co-factors and activators, increases serine and tyrosine phosphorylation of GRIP, respectively resulting in a reduced GluR2/3-GRIP1 interaction. Since PKC activation has been shown to activate Src

(Brandt et al 2003) and an increased PKC translocation and activation has been demonstrated in -prenatal cocaine-exposed brain (Wang et al., 1993), it is possible that cocaine exposure during gestation may increase PKC translocation/activation which in turn phosphorylate GRIP. This hypothesis is affirmed by the data showing elevated levels of membrane-associated typical (α, β and γ) and atypical (δ and ε) isoforms of PKC in the frontal cortices of prenatal cocaine-exposed rats (Figure 3.12). Together with previous report showing that overactive PKC impairs prefrontal cortex-regulated working memory in rats and monkeys (Birnbaum et al 2004), our data support the idea that prenatal cocaine activates PKC by promoting its cytosol to membrane translocation leading to increased PKC and Src mediated GRIP phosphorylation on serine and tyrosine residues which in turn restricts GRIP - GluR2/3 interaction.

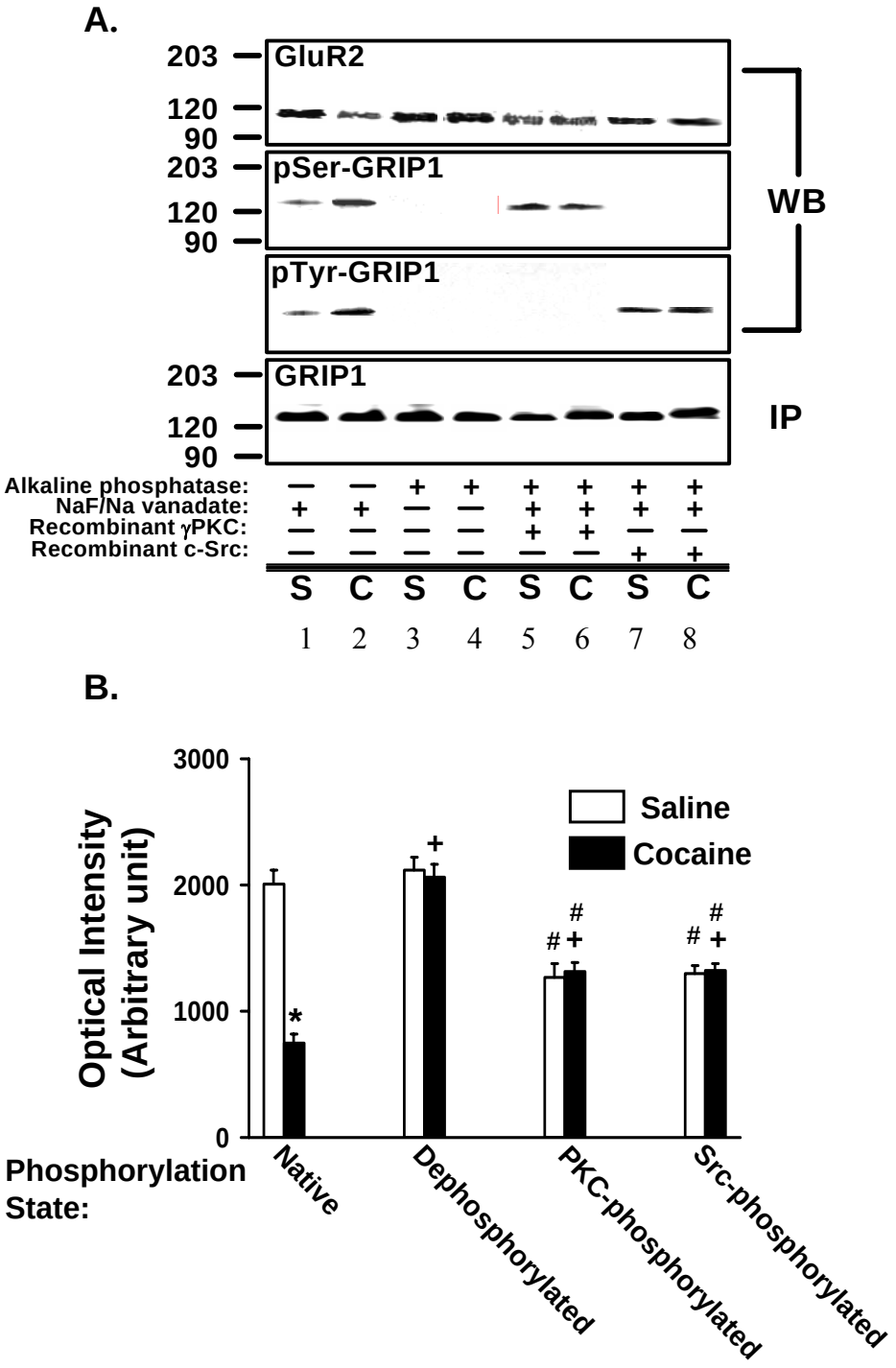

Figure. 3.11. PKC- and Src-mediated phosphorylation of GRIP1 reduces GluR2-GRIP1 coupling in prenatal cocaine exposed rats. (A) Native GRIP1 was purified from frontal cortices of either prenatal saline (**S**)- or cocaine (**C**) -exposed rats by immunoaffinity columns with immobilized antibodies in the presence of protease and protein phosphophatase inhibitors. Purified GRIP1 (10 μg) was treated with vehicle or 100 μg/ml alkaline phosphatase. Following termination of the dephosphorylation by addition of NaF and Na Vanadate, phosphate-free GRIP1 was phosphorylated by recombinant PKC or Src in the presence of ATP in Mg^{2+}-containing Kreb's Ringer,

Following termination of PKC and Src by adding celestrine and PP1, one-half of GRIP1 was analyzed for their phosphor-serine and -tyrosine by Western blotting. The interaction between GluR2 and GRIP1 with different phospohorylation states was assessed *in vitro* by incubation with purified GluR2 from either prenatal saline- or cocaine-exposed rats with 5 μg GRIP1 in 100 μg/ml brain phospholipids, 1% BSA-containing Kreb's-Ringer at 30°C for 30 min with constant shaking. The GluR2-GRIP1 complexes were isolated along with GRIP1 by immunoprecipitation with anti-GRIP1 and the levels of GluR2 associated with GRIP1 determined by Western blotting. The blot is a representative of 4 separate experiments each using a protein preparation from one rat from each group. (B) Densitometric quantification of top blot shown in *A*. $n = 4$. Data are means ± s.e.m. of the optical intensity. The statistical significance was evaluated by Newman-Keuls test for multiple comparisons that followed one-way ANOVA.

*$p < 0.01$ compared to native GRIP1 from saline-treated group.

#$p < 0.01$ compared to dephosphorylated GRIP from both saline- and cocaine-treated groups.

+$p < 0.01$ compared to native GRIP1 from cocaine-treated group.

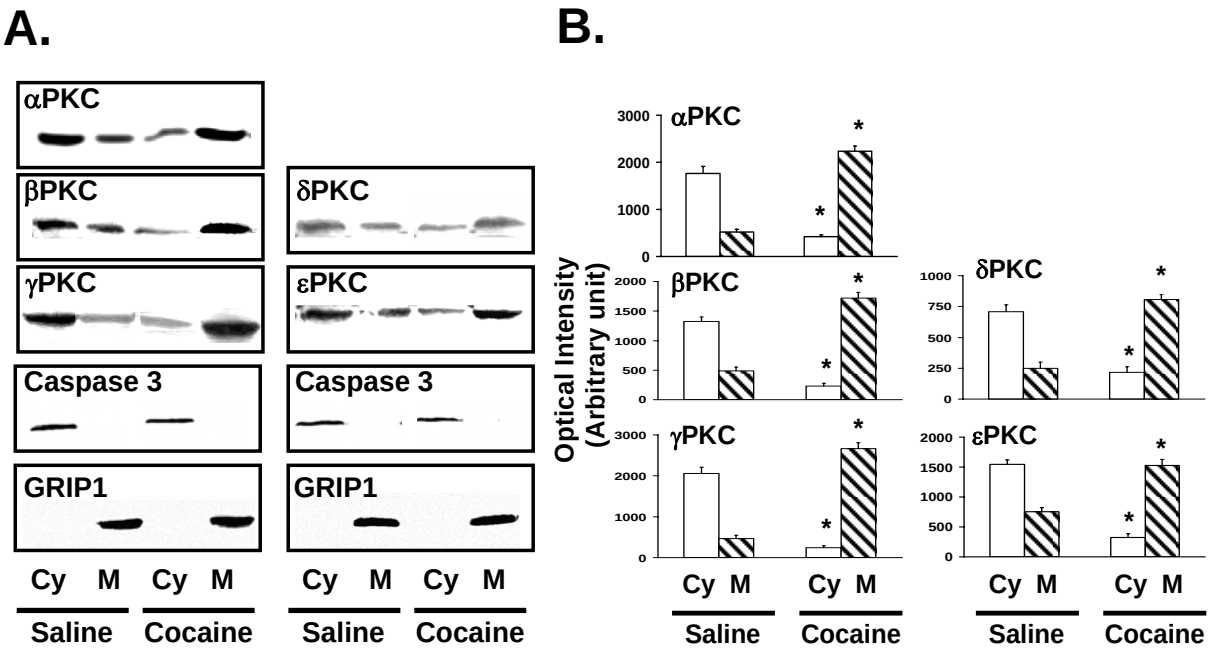

Figure. 3.12: Increased PKC isozymes membrane localization in the frontal cortex of prenatal cocaine-exposed rats. (A) Cytosolic (Cy) and membranous (M) fractions of the synaptosomes were prepared from frontal cortices of 21-day-old rats exposed to saline or cocaine during gestation. Following solubilization by boiling in SDS-containing sample preparation buffer, 50 μg solubilized proteins were size-fractionated on 10% SDS-PAGE and western blots were performed with antibodies against the indicated PKC isozyme (1:500). The blots were stripped and sequentially re-probed with anti-caspase-3 (996 ± 121 and 874 ± 119 optical intensity in saline and cocaine, respectively) and GRIP1 (2349 ± 165 and 2238 ± 151 optical intensity in saline and cocaine, respectively) to validate equal loading in cytoslic and membranous extracts. The blot is a representation of 4 individual rats from each treatment group. (B) Densitometric quantification of blots. $n = 4$. Data are means ± s.e.m. of the optical intensities.

* $p < 0.01$ comparing the level of the indicated PKC isozyme in respective cytosolic and membranous fractions assessed by two-tailed Student's *t*-test.

Prenatal cocaine exposure markedly increases GRASP-1 in the synaptic membrane

Our data illustrating that altered phosphorylation state of GRIP1 in prenatal cocaine-exposed brain could reduce the GRIP1-GluR2 interaction suggest that persistent phsophorylation of GRIPs may alter their interaction with proteins that interact with GRIPs. We further investigate whether the change in the phosphorylation state of GRIP also affects its interaction with GRASP-1, another GRIP associated protein that modulates actin cytoskeleton-regulated trafficking of the GluR2/3 containing AMPARs. GRASP-1, a neuron specific RasGEF, is associated with GRIP/AMPAR complex in brain (Ye et al., 2000). GRASP-1 forms a complex with GRIP1 and AMPARs *in vivo* and the overexpression of GRASP-1 inhibits the synaptic targeting of AMPARs (Ye et al., 2000). We hypothesize that a persistently phosphorylated GRIPs in prenatal cocaine-exposed brains influence GRIP and GRASP-1 interaction which in turn has an impact on the synaptic targeting of AMPARs. To test directly whether prenatal cocaine exposure changes the expression and/or cellular distribution of GRASP-1, the level of GRASP-1 was measured in post-mitochondrial FCX lysate as well as particulate and cytosolic fractions of frontal cortical synaptosomes prepared from 21-day-old prenatal saline- and cocaine-exposed rats by Western blotting. The data summarized in figure 3.13 illustrate that the total expression of GRASP-1 was not altered by prenatal cocaine exposure. In contrast, prenatal cocaine exposure leads to an increased membrane-located concurrent with a reduced cytosolic GRASP-1 level (figure 3.14). Given that the overexpression of GRASP-1 retards AMPAR synaptic expression (Ye et al., 2000), the higher presence of GRASP-1 in the membrane, where it interacts with GRIP, in prenatal cocaine-exposed

brain may suggest the involvement of GRASP-1 in reduced synaptic targeting of GluR2/3 containing AMPARs.

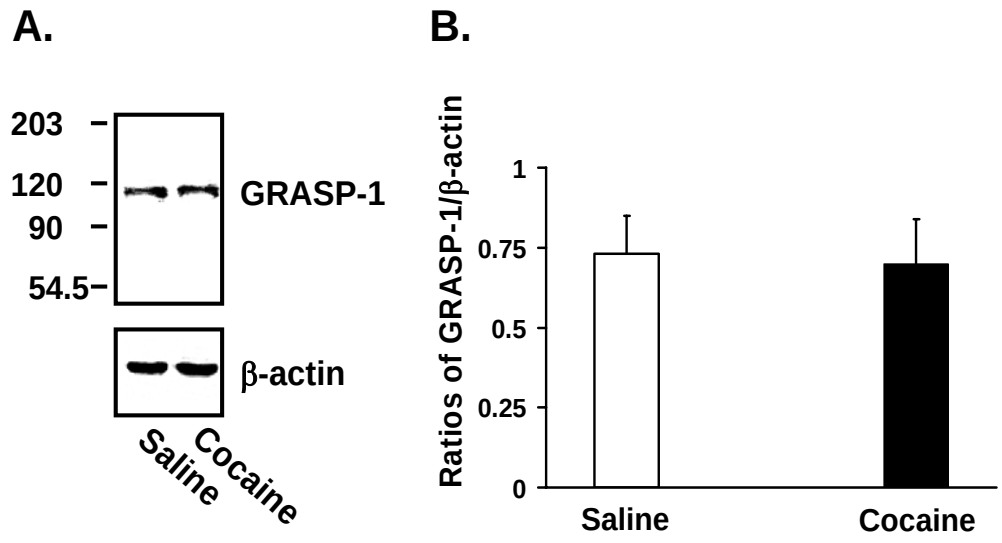

Figure. 3.13: Prenatal cocaine exposure does not alter the expression levels of GRASP-1. (A) The expression levels of GRASP-1 the in post-mitochondrial synaptosome-enriched faction prepared from frontal cortices of 21-day-old rats exposed to saline or cocaine *in utero* were compared by Western blotting. Following solubilization of the brain lysates by boiling in SDS-containing sample preparation buffer, proteins were size-fractionated on 7.5% SDS-PAGE and Western blots were performed with anti-GRASP-1 (1:500). The blots were stripped and sequentially re-probed with anti-β-actin to validate equal loading. The blot is a representative of 4 individual rats from each treatment group. (B) Densitometric quantification of the blot. *n* = 4. Data are means ± s.e.m. of the ratios of the indicated protein to loading control. There were no statistical differences between prenatal saline- and cocaine-exposed rats in these measures.

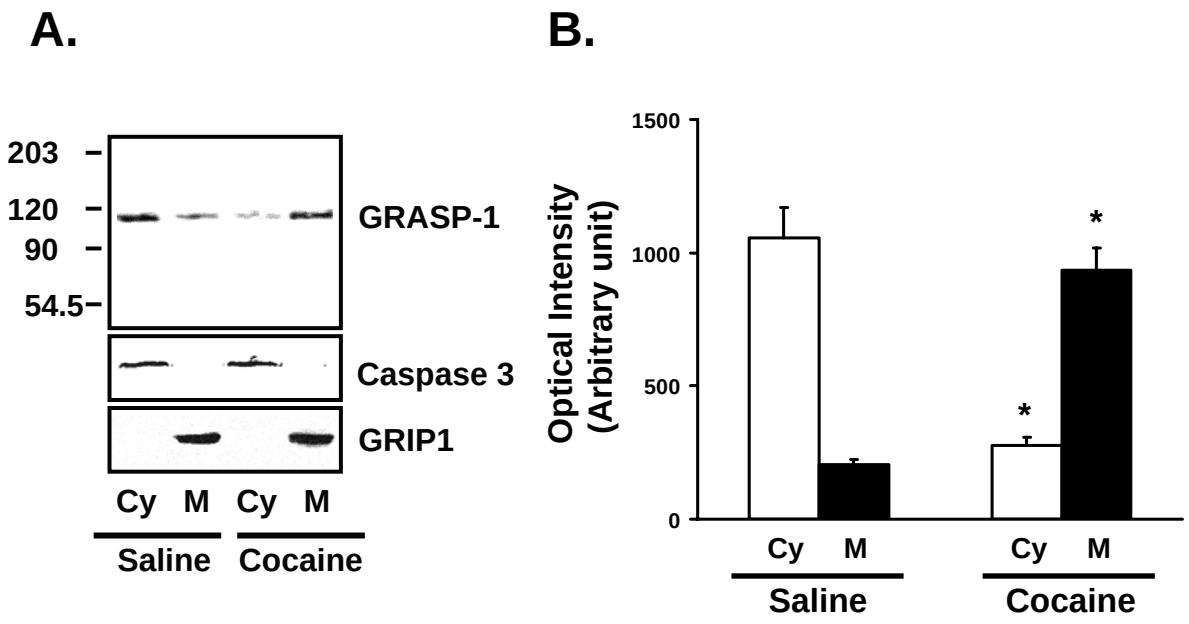

Figure. 3.14: Prenatal cocaine exposure markedly increases the synaptic expression of GRASP-1. (A) Membranous (M) and cytosolic (Cy) fractions of GRASP-1 were prepared from the of FCX of P21 prenatal cocaine- and saline-exposed rats. Following solubilization by boiling in SDS-containing sample preparation buffer, 50µg solubilized proteins were size-fractionated on 7.5% SDS-PAGE and Western blots were performed with anti- GRASP-1 antibody (1:500). The blots were stripped and sequentially re-probed with anti- GRIP1 and -Caspase3 antibodies to validate equal loading in M and Cy extracts. The blot is representative of 4 individual rats from each treatment group. (B) Densitometric quantification of blots. n = 4. Data are means ± s.e.m. of the optical intensity. *$p < 0.01$ comparing the level of GRASP-1 the in cytosolic and membrane fractions.

Prenatal cocaine exposure increases GRASP-1 and GRIP1 association

An increase in membrane associated GRASP-1 may indicate a higher GRASP-1 – GRIP1 interaction in the prenatal cocaine exposed brain. In these set of experiments, the interaction between GRIP1 and GRASP-1 was determined *in vitro*. GRASP-1 and GRIP1 were individually immunoaffinity purified from the FCX of prenatal cocaine and saline treated P21 rat brain. The level of GRASP-1 associated with GRIP1 was determined with western blot using anti GRASP-1, following purification of the GRIP1-associated GRASP-1 by immunoprecipitation with anti-GRIP1 antibody. The western blot (Figure 3.15) shows an increased GRASP-1 and GRIP1 association in prenatal cocaine-exposed compared to the saline-exposed brain. Together with the data showing that GRASP-1 and GRIP1 interaction attenuates the synaptic localization of AMPARs (Ye et al., 2000), the increased GRASP-1 and GRIP1 association in the prenatal cocaine-exposed brain may have contributed to the reduced synaptic expression of GluR2/3-containing AMPARs.

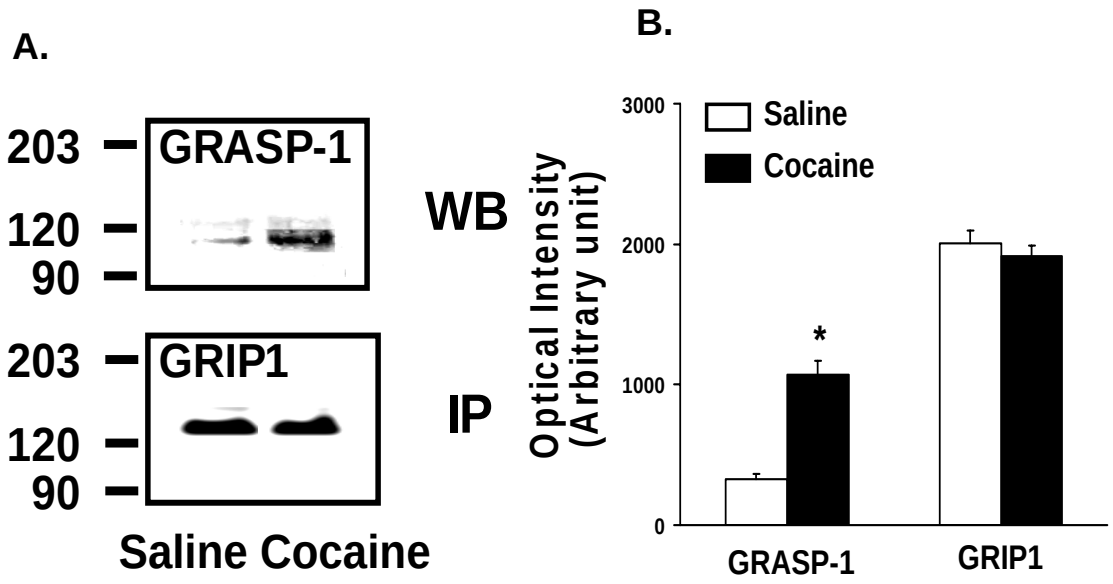

Figure. 3.15: Increased association between GRASP-1 and GRIP1 in frontal cortices prenatal cocaine exposed rats. (A) Native GRASP-1 and GRIP1 were individually purified from frontal cortices of prenatally saline(S)- and cocaine (C)-exposed P21 rat brain by immunoaffinity columns with immobilized antibodies in the presence of protease and protein phosphatase inhibitors. The interaction between GRASP-1 and GRIP1 was assessed *in vitro* by incubating 5μg of each protein in 100μg/ml brain phospholipids, 1% BSA-containing Kreb's-Ringer. The GRASP-1 -GRIP1 complex was isolated along with GRIP1 by immunoprecipitation with anti-GRIP1 and the level of GRIP1-associated GRASP-1 was determined by Western blotting (B) Densitometric quantification of the western blot. n =4. *$p < 0.01$ comparing the level of GRASP-1 with GRIP1 was isolated from prenatal –saline treated rats by Newman-Keuls test for multiple comparisons that followed one-way ANOVA.

PKC and Src-mediated phosphorylation of GRIP1 increases the interaction between GRASP-1 and GRIP1 in prenatal cocaine exposed brain

A heightened PKC and Src-mediated GRIP1 phosphorylation was found to be responsible for the reduced GluR2/3-GRIP1 coupling in the prenatally cocaine-exposed brain (figure 3.11). In light of overexpression of GRASP-1 leads to an increased GRIP1 – GRASP-1 interaction and a reduced synaptic expression of the AMPARs (Ye et al., 2000), we explore the hypothesis that an increased PKC and Src-mediated GRIP1 phosphorylation is also responsible for increased GRASP-1 and GRIP1 association in the prenatal cocaine-exposed brain. We manipulated the phosphorylation state of purified GRIP1 from prenatally cocaine- and saline-exposed FCX using alkaline phosphatase and recombinant PKC or Src in vitro as described previously (figure 3.11). The data presented in figure 3.15 indicate that when the native phosphorylation state of GRIP1 is preserved by adding protein phosphatases, NaF/Na vanadate, an increased interaction between GRIP1-GRASP-1 from prenatal cocaine-exposed brain relative to that of saline-treated controls was observed (figure 3.16 lanes 1 & 2). Dephosphorylating the purified GRIP1 by alkaline phosphatase treatment leads to comparable GRASP-1 –GRIP1 interaction in the prenatal cocaine- and saline-exposed brain (figure 3.16 lanes 7 & 8). As expected, PKC (figure 3.16 lanes 3 & 4) and Src-mediated (figure 3.16 lanes 5 & 6) phosphorylation of previously dephosphorylated GRIP1 increases GRIP1-associated GRASP-1. These results are consistent with the conclusion that PKC and Src-mediated phosphorylation of GRIP1 results in an increased GRASP-1 and a reduced GluR2/3 coupling to GRIP1. GRASP-1 possesses guanine nucleotide exchange factor (GEF) activity (Ye et al., 2000) which influences the activity of various ras-like proteins that

regulate actin cytoskeleton dynamics. Therefore, the altered phosphorylation state of GRIP1 in the prenatal cocaine-exposed brain could shape the interaction between GRIP and cytoskeletal elements thereby influence GluR2/3 trafficking to the synaptic membrane.

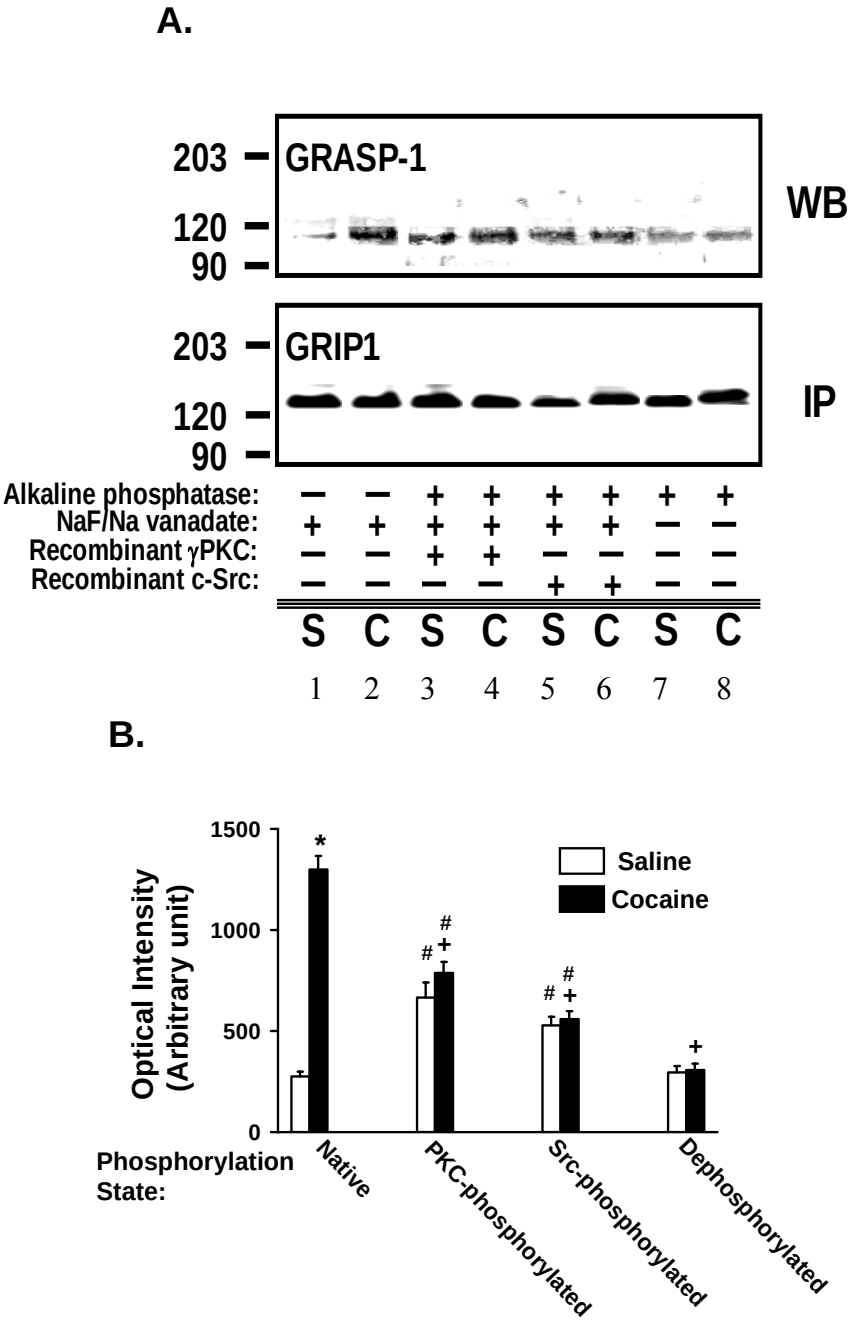

Figure. 3.16: PKC- and Src-mediated phosphorylation of GRIP1 increases GRASP-1 and GRIP1 coupling in prenatal cocaine exposed rats. (A) Native GRIP1 was purified from frontal cortices of either prenatal saline (**S**)- or cocaine (**C**) -exposed rats by immunoaffinity columns with immobilized antibodies in the presence of protease and protein phosphophatase inhibitors. Purified GRIP1 (10 μg) was treated with vehicle or 100 μg/ml alkaline phosphatase. Following termination of the dephosphorylation by addition of NaF and Na Vanadate, phosphate-free GRIP1 was phosphorylated by recombinant PKC or Src in the presence of ATP in Mg^{2+}-containing Kreb's Ringer, Following termination of PKC and Src by adding celestrine and PP1, one-half of GRIP1 was analyzed for their phosphor-serine and -tyrosine by Western blotting. The

interaction between GRASP-1 and GRIP1 with different phosphorylation states was assessed *in vitro* by incubation with purified GRASP-1 from either prenatal saline- or cocaine-exposed rats with 5 μg GRIP1 in 100 μg/ml brain phospholipids, 1% BSA-containing Kreb's-Ringer at 30°C for 30 min with constant shaking. The GRASP-1-GRIP1 complexes were isolated along with GRIP1 by immunoprecipitation with anti-GRIP1 and the levels of GRASP-1 associated with GRIP1 determined by Western blotting. The blot is a representative of 4 separate experiments each using a protein preparation from one rat from each group. (B) Densitometric quantification of the blot. $n = 4$. Data are means ± s.e.m. of the optical intensity. The statistical significance was evaluated by Newman-Keuls test for multiple comparisons that followed one-way ANOVA.

*$p < 0.01$ compared to native GRIP1 from saline-treated group.

#$p < 0.01$ compared to dephosphorylated GRIP from both saline- and cocaine-treated groups.

+$p < 0.01$ compared to native GRIP1 from cocaine-treated group.

Prenatal cocaine exposure increases GRASP-1 RasGEF activity

GRASP-1 contains the catalytic domain of GEFs for the ras family of small G proteins (Ye et al., 2000). To examine whether there were any changes in the RasGEF activity of GRASP-1 in prenatal cocaine treated brains, we measured the RasGEF activity of purified GRASP-1 from prenatal cocaine and saline treated FCX. The release of GDP from ras was measured in the prenatal cocaine and saline treated brains. Recombinant H-ras was loaded with [^{3}H]GDP and then incubated with purified GRASP-1 from prenatal cocaine- and saline-treated brains. In the purified GRASP-1 obtained from prenatal saline treated brain, [^{3}H] GDP released 90% of its radioactivity within 1 minute (figure 3.17). Although prenatal cocaine exposure results in a slower GDP release (2 minutes), the capacity of RasGEF does not seen to be compromised (figure 3.17). This suggests that prenatal cocaine exposure influences the kinetic property but not the capacity of the RasGEF. Because ras-like G proteins such as Rho A, Rac1/Cdc42 and Rap1 are known to regulate cytoskeleton dynamics which in turn affects GluR2/3 trafficking to the membrane, the altered GEF activity in prenatal cocaine-exposed brain may suggest that GRASP-1 contributes to GluR2/3 synaptic expression through manipulation of actin cytoskeleton.

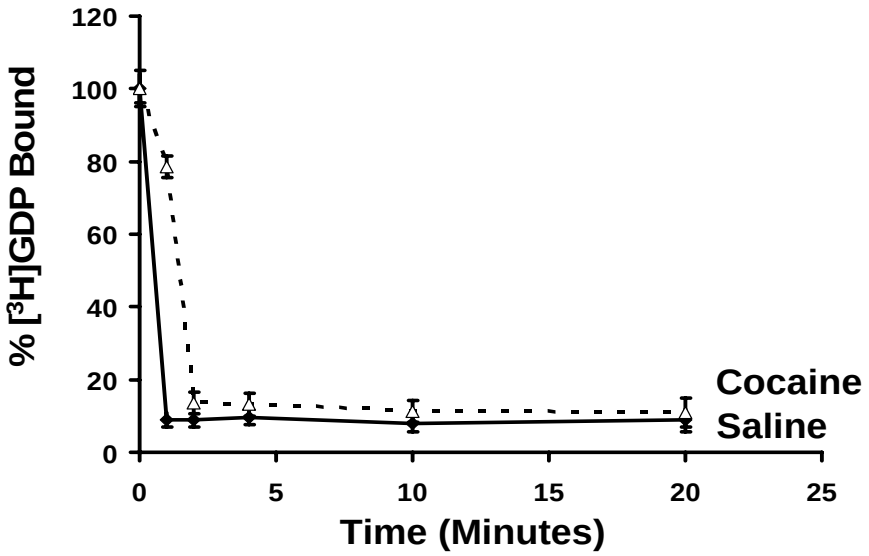

Figure. 3.17: Prenatal cocaine exposure reduces RasGEF activity of GRASP-1 in the FCX. H-ras was loaded with [^{3}H]GDP and then incubated with purified GRASP-1 from the FCX of prenatal cocaine (dashed line) and saline (bold line) treated P21 rat FCX. In GRASP-1 obtained from saline treated FCX H-ras loaded with [^{3}H]GDP lost 90% of its radioactivity within 1 min, while the GDP dissociation of GRASP-1 from prenatal cocaine treated FCX was slower (2 minutes).

Increased activation of small G proteins RhoA, Rap1 and Rac1/Cdc42 in prenatal cocaine exposed brain

The activity of Ras-like G proteins is modulated by RasGEF activity. Change in RasGEF activity is therefore suggestive of an altered activated Ras-like G protein level. Among various ras-like G proteins, RhoA, Rac1/Cdc42 and Rap1 are known to play a role in the trafficking of AMPARs by influencing actin-dependent cytoskeleton. This possibility is supported by several lines of evidence: (1) Ras and Rap have been shown to regulate AMPAR trafficking during LTP and LTD (Zhu et al., 2002), (2) an elevated Rap level reduces the membrane levels of GluR2 (Fu et al., 2007), and (3) Rac1/Cdc42 was shown to induce the clustering of AMPARs (Wiens et al., 2005). Hence, these set of experiments address the question whether the reduced membrane expression of GluR2/3 in the prenatal cocaine exposed brain is the result of changes in the expression and/or activation of the ras-like G proteins including RhoA, Rac1/Cdc42 and Rap1. Rho family of proteins signal to the actin cytoskeleton through a variety of downstream effector proteins which bind specifically to the active (GTP-bound) Rho. Hence, these effector proteins are used as glutathione *S*-transferase (GST) fusion proteins such as in pull-down assays to isolate GTP-bound Rho. Using this method, GTP-bound RhoA was purified from the synaptosomal enriched P2 fraction of the FCX of prenatal cocaine- and saline-treated P21 rats using GST-Rhotekin RBD beads. Similarly, GTP bound Rap1 and Rac1/Cdc42 were pulled down by GST-RalGDS-RBD and GST-Pak1-PBD conjugated beads, respectively from the synaptosomal frontal cortical homogenate of P21 prenatal cocaine and saline treated rats. The levels of GTP Rho1, Rac1/Cdc42 and Rap1 were then determined using Western blotting with specific antibodies directed against each of

ras-like G proteins. The results summarized in Figure 3.18 show a dramatic increase in GTP-bound (active) RhoA, Rap1 and cdc42/Rac1 in the prenatal cocaine-treated rats compared with the saline-treated brain. There were no discernible changes in the expression of the three small G proteins (figure 3.18). Collectively, these results suggest that prenatal cocaine exposure increases active RhoA, Rap1 and cdc42/Rac1. In light of the findings that ras-like G proteins play a pivotal role in actin dynamics, an increased activated RhoA, Rap1 and Rac1/cdc42 may lead to an increased actin polymerization and thereby GluR2/3 trafficking arrest. This hypothesis is supported by data illustring that (1) activation of AMPARs increases RhoA activity leading to an increased actin polymerization (Kim et al 2004), and (2) active Rap1 suppresses synaptic transmission by inhibiting the surface expression of AMPARs containing GluR2 subunit (Fu et al 2007). Recent data presented by Toda et al (2006) indicate that withdrawal from repeated cocaine in adult rat increases actin cycling and actin polymerization in the nucleus accumbens suggesting the role for actin cycling in cocaine addiction. Altogether, our data showing an increased activated RhoA level suggests that prenatal cocaine exposure may increase actin polymerization and filamentous actin (F-actin) level which in turn regulates the synaptic expression of GluR2/3.

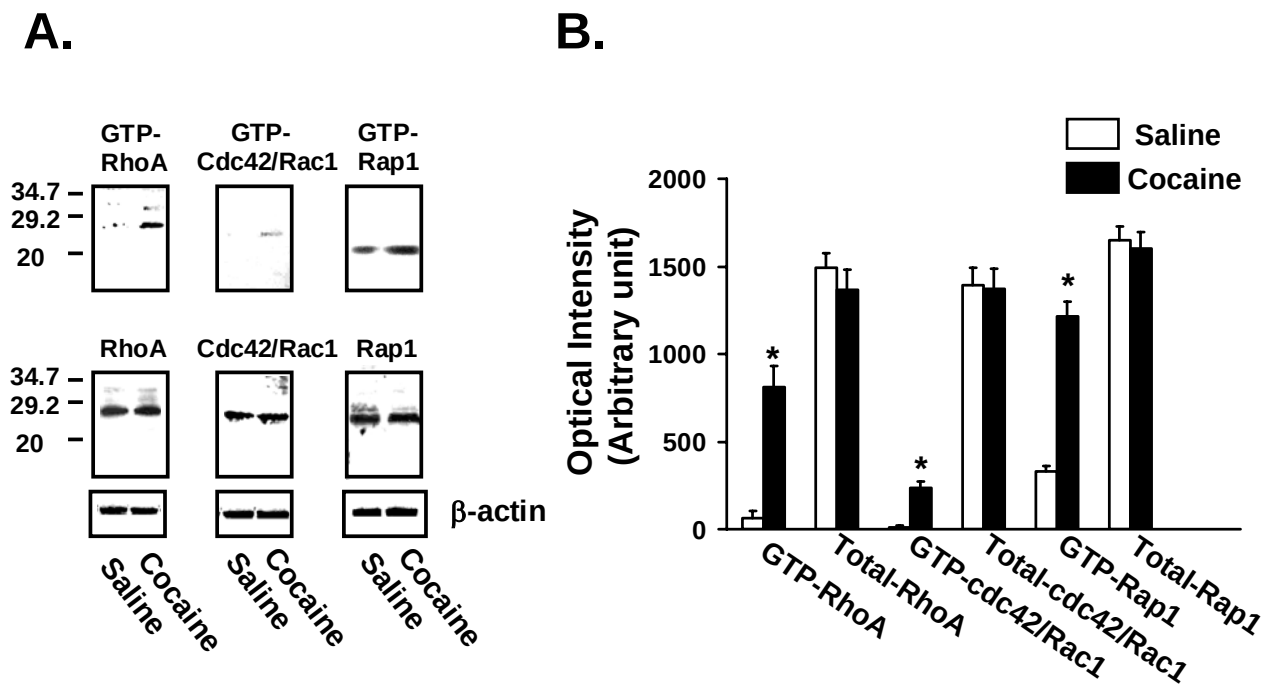

Figure. 3.18: Prenatal cocaine exposure increases activation of ras-like G proteins, RhoA, cdc42/Rac1 and Rap1. Synaptosomes were prepared from the FCX of P21 prenatally cocaine- and saline-treated rats. GTP bound RhoA, Rac1/Cdc42 and Rap1 were isolated from the synaptosomes by GST fusion protein pull down method and their levels were determined using Western blotting with antibodies specific to RhoA, Cdc42/Rac1 and Rap1. In addition, the expression levels of RhoA, Cdc42/Rac1 and Rap1 were also measured by Western blotting with specific antibodies directed against each protein in post-mitochondrial FCX synaptosome-enriched extracts from prenatally cocaine- and saline-exposed P21 rats. In these experiments, blots were stripped and re-probed with anti-β-actin to validate equal loading. (B) Densitometric quantification of blots. n =4. Data are means ± s.e.m. of the optical intensity. * $p < 0.01$ comparing the levels of total protein with the GTP-bound proteins obtained from prenatal cocaine and saline treated groups.

Prenatal cocaine exposure increases F-actin levels

Increase in the levels of active/GTP bound small G proteins has a well documented effect on actin polymerization which can be measured as changes in F-actin levels. Our data showing an increase in activated RhoA, Rap1 and cdc42/Rac1 in the FCX of prenatal cocaine-exposed rats therefore suggest that F-actin level may be altered by *in utero* cocaine exposure. To test this hypothesis, we measured the levels of F-actin in the synaptosomes prepared from the FCX of prenatal cocaine- and saline-treated rat with F-actin labeling probe, rhodamine-conjugated phalloidin. Phalloidin is a toxin which binds more tightly to F-actin subunits, stabilizes and prevents filament depolymerization. Rhodamine, a fluorescent analog bound to phalloidin is used to visualize F-actin levels. The role of phosphorylation on actin cycling was examined by treating FCX synaptosomes obtained from prenatal cocaine- and saline-exposed rats with alkaline phosphatases (1 mg/ml) followed by phosphatase inhibitors. In addition, Cytochalasin D, and potent inhibitor of actin polymerization, was used as an internal control. Actin protein in the synaptosomes was tagged with biotin-conjugated anti-actin following solubilization of the synaptosomes with 0.5% digitonin/0.2% sodium cholate/0.5% NP-40 and dilution. The biotinylated anti-actin linked actin proteins were then immobilized by loading 50% of reaction mixture into each well of the streptavidin-coated 96-well plate. The level of F-actin was then determined using rhodamine-conjugated phalloidin. The fluorescence intensity of the phalloidin labeled samples was detected by a microplate reader (Beckman multimode plate reader, DX880). The data summarized in figure 3.19 shows that prenatal cocaine exposure increases F-actin levels in FCX synaptosomes. The increased F-actin level in the prenatal cocaine-exposed brain is normalized by

alkaline phosphatase treatment (Figure 3.19). In light of the data showing actin filaments can serve as a path for local protein trafficking within the dendritic spine (Kaech et al.,2001; Langford & Molyneaux, 1998) and F-actin serves as an anchor for postsynaptic scaffolding proteins, a change in the actin cytoskeleton observed herein may be suggestive of an altered functional state of postsynaptic proteins, which in turn could affect synaptic efficacy (Allison et al. 1998; Halpain 2000; Lisman & Zhabotinsky 2001; van Rossum & Hanisch 1999). Moreover, our data showing a high level of F-actin in the prenatal cocaine-exposed brain (figure 3.19) is in agreement with an increased activated RhoA, cdc42/Rac1 and Rap1 (figure 3.18) in these brains that favors actin polymerization. Active Rap1 is known to suppress synaptic transmission by inhibiting the surface expression of AMPARs containing GluR2 subunit (Fu et al 2007); the increased Rap1 (and RhoA and Cdc42/Rac1) that we see in prenatal cocaine-exposed brains may indicate a reduced synaptic trafficking of GluR2/3-containing AMPARs.

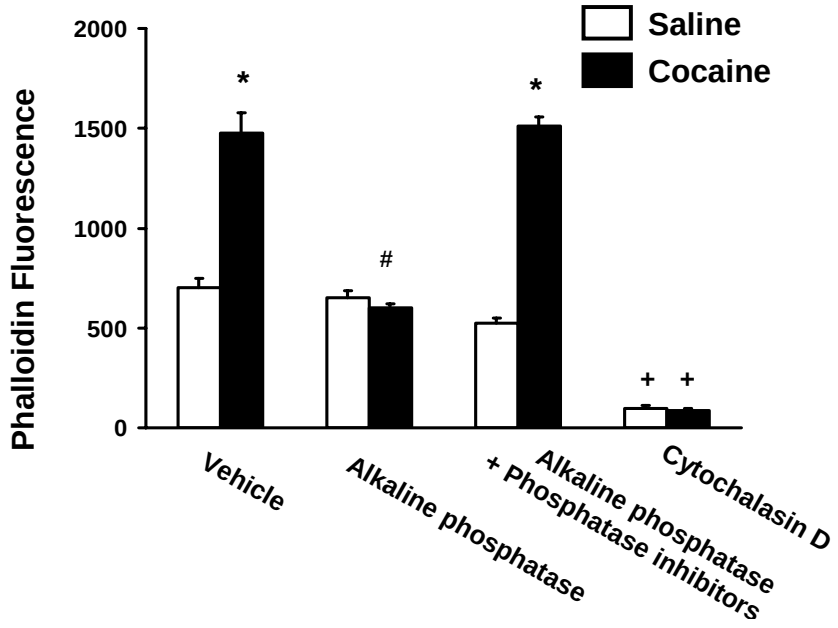

Figure. 3.19: Prenatal cocaine exposure induced an increased F-actin level that is normalized by protein phosphatase treatment. Synaptosomes obtained from the FCX of prenatally cocaine- and saline-treated P21 rats were treated with alkaline phosphatases, alkaline phosphatases (1 mg/ml) plus phosphatase inhibitors or cytochalasin D (10 μM) *in vitro*. Following termination of reaction, synaptosomes were solubilized and actin-containing proteins were extracted using biotin-conjugated anti-actin antibodies, placed into streptavidin-coated 96-well plate in duplication. The level of F-actin was determined by rhodamine-conjugated phalloidin (1 μg/well). After two washes with 100 μl 25 mM Tris, pH7.4 containing 100 mM NaCl, the fluorescence intensity of phalloidin was measured using Beckman multimode plate reader, DX880.

CHAPTER-4

DISCUSSION

The presented data supports the idea that exposure to cocaine during gestation markedly reduces the synaptic incorporation of GluR2 and GluR3 containing AMPARs that cycle on and off the synaptic membranes (constitutive recycling). In accord with the notion, that the constitutively recycling pool of AMPARs is responsible for maintaining the basal synaptic transmission and long-term depression (LTD) (Bredt & Nicoll, 2003), a reduced LTD was observed in the prefrontal cortex (PFC) of 21 day-old prenatally cocaine-exposed rats (figure 3.4). Together with a markedly attenuated synaptic membrane expression of GluR1-containing AMPAR which constitutes the activity-dependent pool of AMPARs (Figure 3.1), the data presented illustrate that prenatal cocaine exposure profoundly dampens AMPAR-mediated neurotransmission in the brain.

The reduced GluR2/3 synaptic expression is the result of reduced coupling between GluR2/3 and their pivotal synaptic scaffolding protein GRIP1/2 (figure 3.8). The reduced GluR2/3 – GRIP association is not caused by changes in the expression of the participating proteins since there are no discernible changes in the expression of GluR2, GluR3 (figure 3.2), GRIP1 and GRIP2 (figure 3.6) in prenatal cocaine- or saline-exposed brains. Similarly, there are no detectable changes in other GluR2/3 interacting proteins including PICK1, ABP and NSF in prenatal cocaine-exposed brains. Although the interaction between PICK1 and GluR2/3 is known to promote Ser^{880} phosphorylated GluR2 and Ser^{891}-phosphorylated GluR3 leading to their dissociation from GRIP (Chung et al., 2000; Kim et al., 2001) and internalization of these AMPA receptors, there are no

detectable changes in pSer^{880}GluR2 and pSer^{891}GluR3 noted in prenatal cocaine-exposed rats (figure 3.5). Together with comparable PICK1 – GluR2/3 association in prenatal cocaine- and saline-exposed brains (figure 3.9), these data collectively support the notion that the reduced GluR2/3 synaptic expression is not the result of an altered PICK1 and GluR2/3 interaction.

Using purified native GluR2/3 and GRIP1 from prenatal cocaine- and saline-exposed FCX in an *in vitro* interaction paradigm, the data showing equally defective interaction between GRIP1 from prenatal cocaine-exposed brain with GluR2/3 from either prenatal cocaine- or saline-exposed brains support the notion that prenatal cocaine exposure has modified GRIPs but not GluR2/3 (figure 3.8). Among various potential mechanisms, we considered the altered phosphorylation state of GRIP as a likely candidate. Although GRIP possesses numerous potential phosphorylation sites including several putative PKC sites, the effect of altered GRIP phosphorylation state on GluR2/3 and GRIP interaction is not known. Our data show for the first time, that prenatal cocaine exposure increases GRIP phosphorylation on serine and tyrosine residues (figure 3.10), suggestive of the involvement of both serine/threonine and tyrosine kinases. The possibility that a persistent GRIP phosphorylation is responsible for the reduced capacity of GRIP to interact with GluR2/3 is further realized by our data showing that dephosphorylation of GRIP1 by alkaline phosphatase restores its capacity to couple with GluR2/3 (figure 3.11). The fact that recombinant PKC and Src phosphorylated GRIP1 leads to a reduced GRIP – GluR2 interaction similar to that observed in the prenatal cocaine-exposed brain leads us to conclude that an enhanced PKC- and Src-mediated GRIP phosphorylation is a prominent mechanism underlying the defected GRIP –

GluR2/3 interaction in prenatal cocaine-exposed brains. The presence of higher membrane-associated typical and atypical PKC isoforms in prenatal cocaine-exposed brains (figure 3.12) further suggests that abnormal persistent activation of PKC in progeny of pregnant cocaine users could result in a deficient AMPAR neurotransmission by causing permanent GRIP phosphorylation. The hyper-activated PKC observed herein is consistent with a previous observation showing that majority of PKC in prenatal cocaine-exposed rabbit brains are membrane-bound (Wang et al., 1993). Together with the demonstration that PKC is capable of activating Src tyrosine kinase (Brandt et al 2004), we provide compelling evidence to show that prenatal cocaine exposure hyper-activates PKC and subset of Src leading to persistent phosphorylation of GRIP underlying the reduced GRIP – GluR2/3 interaction and diminished AMPAR neurotransmission.

Excessive activation of PKC in the prefrontal cortex has been shown to impair cognitive functions, increase distractibility and impulsivity (Birnbaum et al., 2004). It is not clear however, whether such behavioral change is related to disruption of GRIP – GluR2/3 interaction caused by phosphorylation of GRIP and GluR2/3. Nevertheless, by modulating the activity of PKC with agents that interfere with PKC activation by preventing cytosol to membrane translocation without affecting its enzymatic activity may reverse the effects of excessive PKC activation in the prenatal cocaine-exposed progeny. In this regard, lithium and valproate, two therapeutic agents for bipolar disorders that are known to block PKC translocation may be useful in reducing behavioral abnormalities (Wang et al., 2001; Hahn et al., 2005). However, lithium and valproate have been shown to reduce the synaptic expression of AMAPR subunits GluR1

and GluR2 (Du et al., 2003; Gray et al., 2003). On the contrary, studies have shown that chronic treatment with typical antidepressants increases the synaptic membrane expression of GluR1, GluR2 and GluR3 subunits in the hippocampus without the changing their overall expression suggesting an increased AMPAR trafficking (Martinez-Turrillas et al., 2002). Moreover, chronic antidepressant treatment increases the association between the subunits and their synaptic anchoring proteins (non PDZ synaptic scaffolding proteins) that target the subunits to the membrane and restore synaptic transmission (Martínez-Turrillas et al., 2007). Collectively, these reports suggest the possibility that functional abnormalities resulting from reduced synaptic targeting of GluR2/3 in prenatally cocaine-exposed subjects may be rectified by co-treatment with antidepressants and lithium or valproate.

Scaffolding proteins at the excitatory synapses are crucial for the normal operation of synaptic transmission. A reduced interaction between GluR2/3 and GRIP in the prenatal cocaine-exposed brain is therefore suggestive of an altered AMPAR-mediated synaptic transmission. In contrast to our observation, that prenatal cocaine exposure did not alter GRIP and GluR2/3 expression levels, a reduced NMDA receptor scaffolding protein PSD-95 in the striatum was noted in mice that are chronically exposed to cocaine (Yao et al., 2004). Another study also shows that the reduced mGluR1 function following withdrawal from chronic cocaine is associated with a reduced mGluR1-synaptic anchoring protein, Homer in the NAc (Ghasemzadeh et al., 2003). These data collectively indicate that chronic cocaine exposure may alter either the functional state or expression level of scaffolding proteins that are crucial for the clustering of excitatory receptors at the postsynaptic membrane. This view is shared by

Kalivas and Volkow (2005) as they stated in a review article that manipulating the state of scaffolding proteins which, in turn, would alter the synaptic membrane targeting or anchoring of excitatory receptors may serve as a potential pharmacotherapeutic target in cocaine addiction.

Changes in membrane targeting of GluR2/3 in the FCX of prenatal cocaine-exposed rats may also be accompanied by changes in AMPAR receptor trafficking. Among various potential mechanisms, we considered change in the GRIP-associated protein GRASP-1, a neuronal RasGEF that forms a complex with GRIP and AMPARs as a lead candidate (Ye et al 2000). This hypothesis is supported by the data showing an increased GRASP-1 in synaptic membranes of prenatal cocaine-exposed rats (figure 3.14). Like the reduced GluR2 – GRIP1 association in prenatal cocaine-exposed brains, the elevated membrane-associated GRASP-1 (figure 3.14) is also a result of hyper-phosphorylation of GRIP1 (figure 3.16). The overexpression of GRASP-1 has been shown to reduce the synaptic targeting of AMPARs containing GluR2/3 subunit (Ye et al., 2000). It is possible that an increased GRASP-1 – GRIP association (figure 3.15) presents a physical hindrance, thereby preventing an efficient GluR2/3 – GRIP1 coupling although GRASP-1 interacts with the 7^{th} PDZ domain of GRIP and GluR2/3 bind to the 4^{th} and 5^{th} PDZ domain. Alternatively, an increased GRASP-1 and GRIP interaction may cause a conformational change in GRIP obscuring the GluR2/3 binding sites on GRIP.

GRASP-1 possesses Ras guanine nucleotide exchange factor (GEF) activity (Ye et al., 2000), it is therefore possible that prenatal cocaine exposure alters its RasGEF activity without significant effects on GRASP-1 expression since there are no discernible change in GRASP-1 level in prenatal cocaine-exposed FCX (figure 3.13). In support of

this hypothesis, our data show a slower kinetic without alteration in capacity of the GRASP-1 RasGEF activity in the prenatal cocaine-exposed FCX (figure 3.17). Contrasting with the prediction based on a slower RasGEF GDP-GTP exchange that may yield fewer active Ras family G proteins, an increase in active/GTP bound small G proteins RhoA, cdc42/Rac1 and Rap1 was noted in prenatal cocaine-exposed rats (figure 3.18). Although the precise mechanism underlying an increased activated RhoA, cdc42/Rac1 and Rap1 has not been fully elucidated, these data together suggest that an increased GRASP-1 – GRIP association may have placed GRASP-1 in the membrane and hence the vicinity of Ras family G proteins leading to activation of these monomeric G proteins and their downstream effectors. Rap was shown to mediate NMDA receptor-dependent, activity-induced synaptic depression (LTD) by removing AMPARs containing GluR2 or GluR3 from the synaptic membrane (Zhu et al., 2002). Moreover, Rap1 has been shown to suppress synaptic transmission by inhibiting the surface expression of GluR2 containing AMPARs (Fu et al., 2007). In agreement with the latter finding, we observed an increased active Rap1 in the prenatal cocaine-exposed brain concurrent with a reduced synaptic targeting of GluR2/3 containing AMPARs and LTD. In addition to Rap1, active Rac1 can induce clustering of AMPARs in newly formed spines and existing dendritic spines (Wiens et al., 2005). In light of this result, an increase in active Rac1 in the prenatal cocaine-exposed brains may be interpreted as a compensatory mechanism through which the brain may shuttle more AMAPRs to the synaptic membrane or induce the clustering of the existing subunits in the synaptic membrane to restore the failing AMPA mediated synaptic transmission.

In addition to its effects on Rap1 and Rac1 activation, cocaine exposure during gestation also elevates active RhoA level. The Rho family of monomeric G proteins is actively involved in regulating actin cytoskeleton. RhoA is present in postsynaptic density and associated with excitatory glutamatergic receptors at the spine plasma membrane (Schubert et al., 2006). Moreover, the activity of RhoA is dampened by the activation of NMDA and AMPA receptors which in turn restrict actin instability (Schubert et al., 2006). These results suggest that in conditions where NMDA and AMPARs are less active as in prenatal cocaine-exposed brain, there will be a higher level of active RhoA to stabilize actin cytoskeleton and thereby maintain the stability of the postsynaptic structure. Inferring from these data, an increase in active RhoA in the prenatal cocaine-exposed brain may also serve as a compensatory mechanism to facilitate anchoring of the existing pool of AMPARs to the membrane and restoring synaptic transmission via stabilizing the available pool of actin.

Hippenstiel et al (1998) have shown that RhoA and cdc42 activation is required for PKC activation and translocation in human epithelial and endothelial cells. These data suggest the possibility that an increased active RhoA and cdc42/Rac1 may contribute to a higher PKC activation and translocation observed in the prenatal cocaine-exposed brain. In this regard, therapeutic approaches aiming at reducing RhoA and cdc42/Rac activity may help in preventing excessive PKC translocation and activation in the prenatal cocaine-exposed brains. Alternatively, activated PKC may activate Rho which in turn would alter actin cycling, since the activation of PKC in cultured hippocampal neurons has been shown to induce the formation of dendritic lamellae in a Rho/Rac dependent manner (Pilpel & Segal, 2004). These data suggest that the increased

activation of PKC and RhoA in prenatal cocaine-exposed brain may be inter-related. Future experiments will be needed to resolve the cause-effect relationship of the RhoA and cdc42/Rac1 and persistent PKC activation in the prenatal cocaine-exposed brains. Regardless of the sequence of events, the persistent activation of PKC and monomeric G proteins all play a significant role in mediating the defective AMPAR synaptic transmission in prenatal cocaine-exposed brains.

Importantly, hyperactivation of Rac1 and RhoA in hippocampal pyramidal neurons has been shown to disrupt normal spine morphology with pruning dendritic branches (Nakayama et al., 2000). In the anterior cingulated cortex (ACC) of prenatal cocaine-exposed rabbit, dendrites are significantly longer and wavier than their saline-exposed counterparts (Jones et al., 2000). This cocaine-mediated effect seems to be restricted to the dopamine-rich cortical regions such as the ACC and the medial prefrontal cortex since the somatosensory and visual cortex lack such changes (Jones et al., 2000). Similarly, Lloyd et al (2003) have shown changes in the length, volume and extension of dendrites in different layers of the FCX of mice that have had exposure to cocaine during gestation. Together with our findings showing an increased active RhoA, cdc42/Rac1 and Rap1, the possibility that the changes observed in dendritic morphology in the prenatal cocaine-exposed brain is the result of a heightened RhoA, cdc42/Rac1 and/or Rap1 cannot be ignored.

Dendritic spine morphology is known to involve actin cytoskeleton. Together with evidence showing that actin cycling is regulated by the Ras family monomeric G proteins, the increased activated RhoA, cdc42/Rac1 and Rap1 in the prenatal cocaine-exposed brains leads us to speculate that an increase in F-actin (filamentous actin) level

could also occur following *in utero* cocaine exposure. This hypothesis is supported by the data presented in figure 3.19. We further considered the possibility that an increased F-actin level may lead to GluR2/3 trafficking arrest. This theory is in accord with the studies demonstrating that actin cytoskeleton plays a critical role in anchoring neurotransmitter receptors to the postsynaptic membrane. In hippocampal neuronal cultures, incubation with actin depolymerizing, drug latruculin A reduces the number of AMPAR containing spines on pyramidal neurons suggesting that F-actin filaments play a critical role in proper synaptic localization and clustering of AMPARs (Allison et al., 1998). Since AMPARs do not directly interact with the actin cytoskeleton, the interaction of GluR1 (Shen et al., 2000) and GluR4 (Coleman et al., 2003) with actin filaments requires the adapter protein 4.1N, which also regulates the surface expression of these subunits. In contrast to GluR1, the interaction of GluR2/3 containing receptors with actin cytoskeleton has not been fully elucidated. It has been suggested that the interaction of GRIP with other proteins like liprin-α (Wyszynski et al., 2002) and KIF1 (Shin et al., 2003) may link GluR2/3 with microtubules and regulate their synaptic targeting. Collectively, these findings suggest that the trafficking and synaptic anchoring of the AMPARs requires actin cytoskeleton. Hence, the reduced GluR2/3 synaptic expression concurrent with an increased F-actin level in the prenatal cocaine-exposed brains may again suggest that the increased F-actin is a compensatory response to the reduced GluR2/3-mediated function through which prenatal cocaine-exposed brain is making an attempt to restore proper density of AMPARs in the synaptic membrane. Alternatively, the increased F-actin in the synaptic membranes of prenatal cocaine-exposed brains may represent means to stabilize the available pool of AMPARs to the postsynaptic membrane

in order to restore the synaptic transmission. Lastly, the increase in F-actin levels seen in prenatal cocaine-exposed rats could also be an effect of withdrawal from cocaine, since an increased F-actin level was observed in adult rat brains following 3 weeks of withdrawal from repeated cocaine (Toda et al., 2006).

In this work, for the first time, we obtained clear evidence showing changes in the synaptic expression of AMPAR subunits GluR1, GluR2 and GluR3 in the prenatal cocaine exposed FCX. Since the synaptic membrane expression of GluR2/3 containing AMPARs is critical in maintaining the basal excitatory synaptic transmission as well as LTD, our results showing a reduced LTD in the prenatal cocaine-exposed brain indicates an altered synaptic plasticity in these animals. These findings are in agreement with data showing that a reduced synaptic localization of constitutive recycling pool of AMPARs result in a reduced LTD (Carroll et al., 1999; Man et al., 2000). It is also possible that the reduced LTD may be a consequence of withdrawal from *in utero* cocaine. This view is supported by data illustrating a decreased maximal amount of LTD in the NAc slices from mice withdrawn from cocaine (Thomas et al., 2001). The attenuated synaptic membrane expression of GluR2/3 containing AMPARs with corresponding reduction in LTD in prenatal cocaine-exposed brains implies that an altered reward property in offspring of pregnant cocaine users may be mediated in part by a reduced AMPAR activation. In light of the facts that pharmacological inhibition of the AMPARs ((Kaddis et al., 1995; Harris & Aston-Jones, 2003; Backstrom and Hyytia, 2003)) as well as GluR1 and GluR2 knockdown (Dong et al., 2004; Mead et al., 2005) both attenuate brain stimulation, cocaine and food reward, the reduced synaptic recruitment of GluR2, GluR3 and GluR1 in prenatal cocaine-exposed brains may suggest that tolerance to addictive

drugs may have developed in the offspring of pregnant cocaine users. This hypothesis is supported by the data demonstrating that rabbits that have had exposure to cocaine in utero are tolerant to cocaine-induced motor sensitization, an indicator of addictive tendency to cocaine (Stanwood & Levitt 2003; Thompson et al., 2005). The reduced AMPAR neurotransmission in frontal cortex that commends the executive function in prenatal cocaine-exposed rats may predict a poorer frontal brain-driven cognitive function in these *in utero* cocaine-exposed subjects. This hypothesis is collaborated by a recent demonstration that children (mean age 10 yrs) who have had exposure to cocaine during gestation show poor executive functioning and a less mature development of frontal pathways (Warner et al., 2006). A cohort study also shows that children prenatally exposed to cocaine are at a very high risk for developing learning disabilities or impaired intellectual functioning by age seven (Morrow et al 2006). These findings suggest that prenatal cocaine-exposed progeny exhibit impairments in cognitive functioning that is more apparent with advancing age. Although a reduced AMPAR neurotransmission is likely involved in the abnormal cognitive processes like learning and memory in prenatal cocaine-exposed subjects, various adaptive changes in response to the reduced GluR2/3 recycling in these brains including changes in synaptic transmission mediated by the metabotrophic glutamate receptors (mGluRs) and/or glutamate release due to alterations in synaptic vesicle transport system at the presynaptic terminal (data not shown), are most certainly involved. Although we illustrate a deficient AMPAR system in prenatal cocaine-exposed rats at the age when AMPARs are fully matured (Zhu et al., 2000), we have not elucidated whether such a defect in this prominent excitatory brain system is permanent. Future research efforts will resolve this

issue by determining GluR2/3 synaptic expression in brains of prenatal cocaine-exposed rats at various ages.

Collectively, our data presented here and reports by many laboratories demonstrate that prenatal cocaine exposure alters the structure and function of the brain in a number of ways. Such functional abnormalities are evidenced in studies conducted in both human and animal models indicating that prenatal cocaine exposure results in deficits in the brain development, attention processing, motor skills, and language skills that may persist into adulthood (Delaney-Black et al., 1996; Mayes et al., 1995; Chiriboga et al., 1993; Bandstra et al., 2002). Adult rabbits that have had exposure to cocaine during gestation show abnormal motor function, associative learning and discrimination learning (Romano et al., 1995; 1996). Behavioral changes are also accompanied by a sustained change in brain morphology, structure and organization in the affected brain areas in the prenatal cocaine exposed progeny. This is illustrated by the data showing that prenatal cocaine exposure produces persistent changes in the dendritic length, dendritic volume and shape of pyramidal neurons in the cerebral cortex (Stanwood et al., 2001; Lloyd et al., 2003; Jones et al., 2001). Most of the work done so far in this area has also pointed to the role of dopamine receptor dysfunction (Wang et al., 1995; Friedman et al., 1996; Zhen et al., 2001), structural changes in specific GABAergic neurons (Morrow et al., 2003), and changes in norepinephrine system (Booze et al., 2006). Consistent throughout the above-mentioned studies is that some deficits in learning and cognitive processes are observed in subjects who have had exposure to cocaine during gestation. Despite the overwhelming evidence showing that

glutamatergic systems are vitally important to properly execute complex cognitive processes, few studies thus far have investigated whether prenatal cocaine exposure influences glutamatergic receptors, in particular the AMPARs that mediate the majority of excitatory neurotransmission in the brain. The fact that dopaminergic and the glutamatergic pathways co-localize and interact with each other in multiple brain areas including the reward circuitry suggests that alterations made on glutamate receptors by drugs of abuse could contribute to ultimate brain changes following drug usage. The reward pathways that are involved in drug addiction include (i) the mesolimbic pathway which originates from the dopamine cells of the ventral tegmental area (VTA) and innervates the nucleus accumbens (NAc) regulates mood and reward and (ii) the mesocortical pathway that arises in the VTA and innervates the frontal cortical structures, which are implicated in working memory, attention, impulse control and other aspects of executive function. The VTA sends dopaminergic projections to both the NAc and the prefrontal cortex; whereas prefrontal cortex sends glutamatergic inputs to both the VTA and the NAc. This reciprocal innervation pattern leads to the hypothesis that dopamine is a key mediator in acute reward and in the initiation of addiction whereas end-stage addiction is a result of cellular adaptations in the prefrontal glutamatergic innervation (Kalivas and Volkow (2006). In support of this theory, Boudreau & Wolf (2005) have demonstrated that an increased surface expression of AMPARs GluR1 and GluR2/3 in the NAc is closely associated with the magnitude of behavioral changes in cocaine-sensitized rats. Together with reports showing that repeat extinction training after repeated self-administration of cocaine leads to an up-regulation of GluR1 and GluR2/3 in the NAc (Sutton et al., 2003; Self et al., 2004), these data support the notion that

cocaine abuse results in changes in glutamatergic AMPARs in the adult brain. Collectively, these data and our results provide compelling evidence to show that cocaine exposure to developing and adult brain results in substantial and possibly long-lasting changes in AMPAR glutamatergic system that shape the brain function.

It must be noted that in addition to the defected AMPARs, prenatal cocaine exposure has also been shown to affect numerous neurotransmitter systems including dopamine D_1 receptor – $G_{s/olf}$ coupling (Wang et al., 1995; Friedman et al., 1996; Jones et al., 2000; Zhen et al., 2001), GABAergic neurons (Wang et al., 1995) and noradenergic system (Booze et al., 2006). Among them, the defected D_1 receptor signaling appears to be most relevant to the reduced AMPAR synaptic membrane localization since D_1 receptor stimulation was shown to recruit AMPARs to synaptic membrane (Ngiavacchi & Wolf 2004). However, it is not clear whether the observed reduction in AMPAR synaptic expression in prenatal cocaine-exposed brains is the result of defected D_1 dopamine receptor. Although the contributions from each of the altered neurotransmitter system in prenatal cocaine-exposed brain to neuronal dysfunctions and behavioral abnormalities have not yet been clearly defined, our data presented here provide compelling evidence to show that prenatal cocaine exposure results in persistent membrane localization and activation of PKC (and Src kinase). We further demonstrate that this hyper-activated PKC could lead to a sustained phosphorylation of GRIPs and reduced GluR2/3 synaptic membrane targeting. It is unclear, however, whether changes in GRASP-1 localization in prenatal cocaine-exposed brains contributed to the reduced GluR2/3 – GRIP interaction or rather serve as a compensatory mechanism to minimize the impact of AMPAR deficiency.

In conclusion, we present evidence to show that a reduced GluR2/3 synaptic expression may be the mechanism underlying the defected AMPAR-mediated synaptic plasticity (LTD). We further illustrate that a persistent phosphorylation of GRIP mediated by the hyper-activated PKC is a prominent causal factor of the reduced GluR2/3 – GRIP and increased GRASP-1 – GRIP interactions. Given that AMPARs mediated the majority of the brain excitatory neurotransmission underlying complex cognitive behaviors, our data suggest that the deficient AMPAR signaling in prenatal cocaine-exposed brain is a prominent mechanism underlying cognitive impairments of these subjects. Since our data on the reduced GluR2-, GluR3-containing AMPARs were collected in brains from 21-day-old prenatally cocaine-exposed rat, a longitudinal study that follows the changes in the AMPAR neurotransmission in brain at various postnatal ages will be needed to elucidate whether defected AMPAR synaptic expression is a persistent event.

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