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DOPAMINE-INDUCED SYNAPTIC PLASTICITY IN THE AMYGDALA IN SALINE-AND COCAINE-TREATED ANIMALS UNDERGOING CONDITIONED PLACE PREFERENCE

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by

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For my parents, who have always supported me.

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DOPAMINE-INDUCED SYNAPTIC PLASTICITY IN THE AMYGDALA IN SALINE-AND COCAINE-TREATED ANIMALS UNDERGOING CONDITIONED PLACE PREFERENCE

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One of the biggest problems facing cocaine addicts is relapse and currently there are no treatments for relapse. These studies will provide insight into the underlying mechanisms of LTP mediating relapse to cocaine. A major mechanism of cocaine in addiction is to inhibit dopamine re-uptake and glutamate is an excitatory neurotransmitter involved in addiction. Previous studies have demonstrated a link between phospholipase D (PLD) and metabotropic glutamate receptors (mGluRs), while other studies show an interaction between dopamine receptors (DRs) and PLD. Therefore, we investigated the mechanisms of the dopamine agonist-induced synaptic plasticity that is enhanced in the amygdala due to cocaine exposure in the Central-Basolateral Amygdala (CeA-BLA) pathway. Electrophysiology recordings showed that the dopamine agonist-induced LTP is mediated via D1 receptors and is dependent upon mGluR1 and the mGluR linked to PLD and partially dependent upon mGluR5 and phospholipase C (PLC). Western blots and co-immunoprecipitations revealed increased expression of PLD after cocaine administration and possible physical interactions between group I mGluRs and PLD and DRs and PLD. PLD activity assays showed that PLD activity was decreased by antagonists of mGluR1 and the mGluR linked to PLD and was increased by the agonist for the mGluR linked to PLD. Basal PLD activity may also be mediated by D1 receptors and was not affected by mGluR5 antagonists.

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List of Abbreviations and Drugs

ACPD; (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate; nonselective mGluR agonist

AIDA; 1-aminoindan-1,5-dicarboxylic acid; group I mGluR agonist

BLA; Basolateral amygdala

CeA; Central amygdala

CPP; Conditioned place preference

DAG; diacylglycerol

DHPG; (RS)-3,5-dihydroxyphenylglycine; selective group I agonist

fEPSP; field excitatory synaptic potential

HFS; high frequency stimulation

L-CSA; L-cysteine sulfonic acid; selective agonist of the mGluR linked to PLD

LTP; long term potentiation

LY367385; selective, competitive mGluR1 antagonist

MPEP; 2-methyl-6-(phenylethynyl)-pyridine; non-competitive mGluR5 antagonist

MCPG; methyl-4-carboxyphenylglycine; competitive antagonist for group I mGluRs

PCCG-13; (2R, 1'S, 2'R, 3'S)-2-(2'-carboxy-3'-phenylcyclopropyl)glycine; selective antagonist for the mGluR linked to PLD

PLC; Phospholipase C

PLD; Phospholipase D

PTX; picrotoxin; noncompetitive $GABA_A$ antagonist

Raclopride; selective D2-like antagonist

SKF81297; selective D1 agonist

SCH23390; selective D1 antagonist

U-73122; selective PLC antagonist

BACKGROUND

Cocaine Addiction

Cocaine is a national health problem. The 2005 National Household Survey on Drug Use and Health (NSDUH) reported that 112 million Americans over the age of eleven (46% of the population) have used illicit drugs at least once in their lifetime while 8% have used drugs at least once in the past month (Substance Abuse and Mental Health Services Administration, 2007). A 2002 NSDUH survey of cocaine usage reported that cocaine initiation steadily increased from 1990 to 2000, peaking at 1.2 million in 2001. Nearly a quarter of Americans age 26 to 34 have used cocaine in their lifetime and 1.5 million people are classified as being addicted to cocaine (Substance Abuse and Mental Health Services Administration, 2007).

Addiction is considered a chronic, relapsing disorder in which craving and drug seeking relapse occur even after drug abstinence (Gawin and Kleber, 1986). The biggest problem with the treatment of drug addiction is the inability to treat the high propensity towards relapse (O'Brien, 2005). Relapse after prolonged absence from a drug can occur on re-exposure to the drug, drug-associated cues, and stress, all factors that induce increased craving in humans (O'Brien et al., 1998; Sinha et al., 2000; Sinha, 2001). Current pharmacotherapy, or detoxification, for cocaine, heroin, and opiate addiction can successfully remove craving during treatment and shortly after, but this therapy is not a permanent solution (O'Brien, 2005). This treatment consists of giving the patient a similar drug to the one currently causing addiction and treating the side effects while slowly weaning the patient from the new drug, such as naltrexone for opiate addicts (O'Brien, 2005). The fact that these treatments are only sufficient in alleviating short-term effects and not addiction itself suggests that long-term neuroadaptations take place during addiction which can result in relapses after long periods of time and can be referred to as a "drug-induced neural plasticity" (Nestler et al., 1993; Nestler, 2001). Because these neuroadaptations make treatment extremely difficult, understanding their underlying mechanisms is an essential element in addressing a cocaine addiction.

Conditioned Place Preference

Conditioned place preference (CPP) is a classical conditioning procedure (Shalev et al., 2002). In this paradigm, a drug is associated with contextual stimuli (visual, tactile, auditory, or olfactory); this association can result in drug-induced conditioning where the animal associates these cues with the drug. This paradigm is used to quantify the association between the drug administered and the cues paired with the drug (Carr et al., 1998). The time animals spend on the drug-paired side of a chamber is compared to amount of time spent on that side during baseline recordings. If more time is spent on that side after drug pairing, CPP has occurred (Carr et al., 1989).

Amygdala and cocaine

Amygdala lesions block the context-association of CPP (Brown and Fibiger, 1993) and the basolateral amygdala (BLA) is necessary for cue-induced return to drug use (Meil and See, 1997). Limbic regions including the amygdala of cocaine patients showed increased cerebral blood flow when presented with videos of cocaine cues; these patients also exhibited signs of craving in response to the presented cues (Childress et al., 1999). Stimulation of the BLA complex at a 20 Hz frequency can re-instate cocaine seeking in rats (Hayes et al., 2003). The same stimulation in other brain regions does not result in cocaine seeking re-instatement. The reinstatement animal model used to study relapse has shown that the BLA complex is a main component of the neural circuitry responsible for conditioned cue-reinstatement of cocaine seeking behavior (Meil and See, 1997; Ciccocioppo et al., 2001; Kantak et al., 2002; McLaughlin and See, 2003). The current project uses as a model the association of contextual cues and drug administration in CPP to analyze the circuits in the amygdala involved in the processing of cue information.

Anatomy of BLA- CeA pathway

The BLA receives input projections from the medial prefrontal cortex (mPFC) (Otterson, 1989; McDonald et al., 1996) and it has been proposed that predominating flow of information is from the BLA to the central amygdala (CeA) (Pitakanen et al., 1997). The BLA contains pyramidal-like projection neurons and inhibitory interneurons (McDonald, 1985; 1992). Along with the "classical" interneurons found within the BLA, one group has recently shown that there is also another type of smaller interneuron found along the external capsule, lateral paracapsular cells, (lpcs) and those more medial cells (mpcs) between the BLA and CeA (Marowsky et al., 2005) **(Illustration 1)**. The mpcs and lpcs are anatomical markers as well as different types of interneurons.

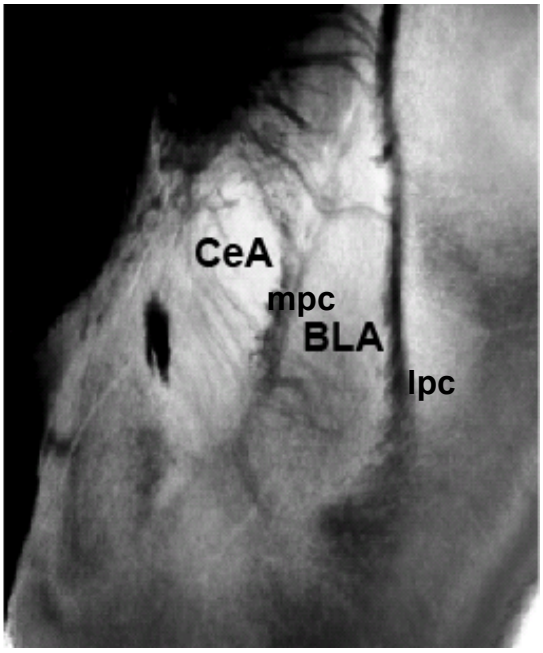

Illustration 1: Interneuron subtypes in the amygdala. Depicts location of the medial paracapsular cells between the BLA and CeA and the lateral paracapsular cells along the external capsule. The mpcs are the ones that play a major role in our experiments.

Dopamine and Cocaine

Dopamine receptors (DRs) are directly coupled to G-proteins (GPCRs) and are divided into two categories: D1-like receptors, including D1 and D5 subtypes, and D2-like receptors, including D2, 3, and 4 subtypes. Both D1-like subtypes stimulate

adenylate cyclase via G_s and G_{olf} (Sibley and Monsma, 1992) but D5 receptors are ten times more receptive to dopamine than D1 (Missale et al., 1998). D1-like receptors also stimulate the G-protein coupled to phospholipase C (PLC) which catalyzes breakdown of phosphatidylinositol (PI) into inositol 1,4,5-triphosphate which then releases calcium from intracellular stores. D2-like receptors can have the opposite effect of D1-like receptors and inhibit adenylate cyclase. D2-like receptors inhibit voltage-gated calcium currents (Lledo et al., 1992) and activate potassium currents (Einhorn et al., 1991). These effects are both mediated through inhibition of adenylate cyclase via G_i/G_o. In the amygdala, D1-like receptors have been shown to be linked to PLC not adenylate cyclase (Undie and Friedman, 1990; Leonard et al., 2003).

Dopamine levels in the amygdala are increased after one month of cocaine withdrawal (Tran-Nguyen et al., 1998), which suggests that dopamine plays a role in the neuroadaptions resulting from chronic cocaine administration. More specifically, dopamine levels in the BLA are increased during learning (Hori et al., 1993) and due to stressful or predictive stimuli (Harmer and Philips, 1999; Inglis and Moghaddam, 1999). Dopamine projections densely innervate the BLA (Brinley-Reed and McDonald, 1999) and both D1-like and D2-like receptor types are present in the amygdala (Scibilia et al., 1992).

D2 receptors block feedforward GABAergic inhibition in the lateral amygdala pathway which may enable induction of long-term potentiation (LTP) (Bissiere et al., 2003). DA enhanced postsynaptic firing of projection neurons and local interneurons within the BLA via D1 receptors (Rosenkranz and Grace, 1999). Some studies showed that BLA interneurons are depolarized by D1 activation, thereby increasing inhibition (Kroner et al., 2004; Rosenkranz and Grace, 2002). However, another group stated that the balance of excitatory and inhibitory projections throughout the BLA to increased excitation in the BLA-CeA pathway is due to D1 activation (Pape, 2005) **(Illustration 2)**. This is likely explained by dopaminergic hyperpolarization of medial interneurons between the BLA and CeA pathway (Marowsky et al., 2005).

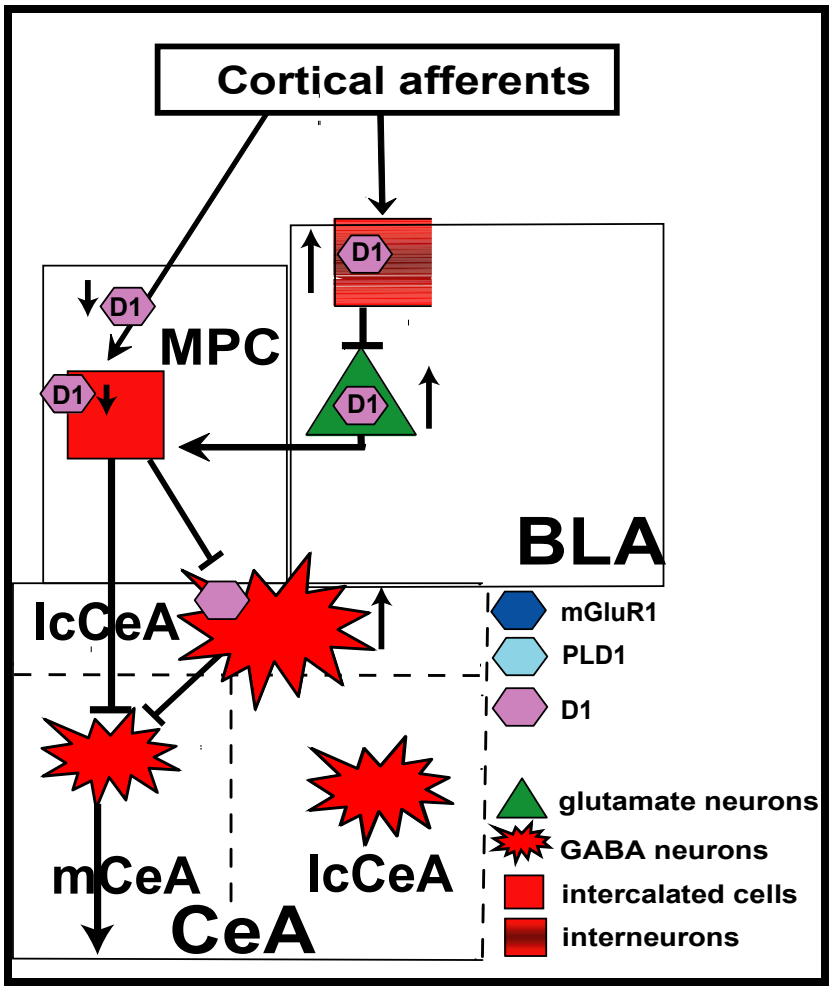

Illustration 2: Excitation in the BLA-CeA pathway. Shows that excitation of DRs on BLA projection neurons results in excitation in the CeA via decreased inhibition from GABA interneurons.

Administration of the D1 antagonist, SCH23390, blocked reinstatement of cue-induced cocaine seeking behavior (Berglind et al., 2006) whereas low doses of the D2 antagonist, raclopride, did not block reinstatement. Higher dose of raclopride, however, did attenuate the relapse to cocaine seeking. However, the reported effects of DR antagonists on cocaine CPP are not consistent. The D1 antagonist, SCH23390, did not block the expression of cocaine CPP (Cervo and Samanin, 1995) but prevented CPP acquisition (Cervo and Samanin, 1995; Pruitt et al., 1995; Nazarian et al., 2004). In

contrast, D2 antagonists blocked the acquisition of cocaine CPP while a combination of D1 and D2 antagonists blocked cocaine CPP expression (Liao et al., 1998).

MGluRs

Glutamate, like dopamine, plays a major role in cocaine addiction and long-term neuroplastic changes that lead to relapse via cue-induced reinstatement (Kalivas, 2004). Metabotropic glutamate receptors, mGluRs, are one of two major types of glutamate receptors in the central nervous system (Pin and Duvosin, 1995). The other type is ionotropic glutamate receptors, AMPA, NMDA, and kainate, the subunits of which form ion channels that allow cations to cross the membrane once glutamate binds (Pin and Duvosin, 1995). There are three groups of mGluRs based on sequence homology, signal transduction mechanisms, and pharmacological mechanisms: Group I (mGluRs 1 and 5), Group II (mGluRs 2 and 3), and Group III (mGluRs 4, 6, 7, and 8) (Ferraguti and Shigemoto, 2006). Group I mGluRs are positively linked to the $G_q/_{11}$ type of G-protein which activate adenylate cyclase and phospholipase C and form inositol 1,4,5 trisphosphate and diaceyl glycerol (Abdul-Ghani et al., 1996). The other two groups are negatively linked to G_i/G_o which results in the inhibition of adenylate cyclase.

Group I mGluRs (mGluR1 and mGluR5) play a major role in addiction (Swanson et al., 2001). After one week of repeated cocaine administration, mGluR1-induced glutamate release in the nucleus accumbens is attenuated and Homer1 protein levels are decreased (Swanson et al., 2001). Homer1 acts as an intracellular scaffolding protein which regulates mGluR signaling (Tu et al., 1999). Homer1b/c, in particular, links mGluR1 and mGluR5 with IP3 receptors and iGluRs via additional scaffolding proteins which makes it an important component of the excitatory post-synaptic density (Naisbitt et al., 1999). It has also been shown that mGluR5 mediates cocaine enhanced locomotor activity and the drug's rewarding properties (Chiamulera et al., 2001).

It is well known that mGluRs play a major role in mental processing within the amygdala that can be seen at both the molecular and behavioral levels. In a model of knee-joint arthritis pain, group II and III mGluRs inhibit nociceptive input to neurons in

the laterocapsular division of the central nucleus (CeLC) of the amygdala (Li and Neugebauer, 2006). Fear-potentiated startle tests showed that infusions of the mGluR II agonist, DCG-IV, into the amygdala blocked consolidation of fear memory (Lin et al., 2007). Additionally, injections of the mGluR2/3 antagonist, LY379268, into the central amygdala reduced the expression of incubated cocaine craving (Lu et al., 2006). Group I mGluRs also play a role in amygdala functions. Amygdalar administration of LY456236, an mGluR1 antagonist, decreased behavioral and electrographic seizures at threshold stimulus intensity in kindled rats (Shannon et al., 2005). Furthermore, CPCCOEt (7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester), an mGluR1 antagonist, blocked extinction, but not acquisition, of fear conditioning when injected into the lateral amygdala forty eight hours after fear conditioning initiation (Kim et al., 2007). Additionally, mGluR5 receptors play a role in modifying fear and anxiety in amygdala because injections of MPEP, a selective mGluR5 antagonist, into the rostral area of the amygdala, near the intercalated cells at the junction between the BLA and CeA, results in anxiolytic behavior (de al Mora et al., 2006). MGluR1 has also been shown to mediate epilepsy. The mGluR1 antagonist, LY456236, was able to decrease behavioral and electrographic seizures in amygdala-kindled rats (Shannon et al., 2005). Taken together, mGluRs mediate multiple functions within the amygdala, including cocaine craving. Thus, we investigated the role of group I mGluRs in the BLA-CeA pathway after withdrawal from chronic cocaine.

Phospholipase D (PLD)

PLD is an enzyme, which controls numerous biological functions including exocytosis, phagocytosis and membrane trafficking. It catalyzes the transphosphatidylation reaction which hydrolyzes the phosphodiester bond of phosphatidylcholine forming phosphatidic acid and diacylglycerol (DAG), releasing choline as a byproduct. It is activated by numerous endogenous molecules, including neurotransmitters, hormones, and growth factors.

There are two known PLD isoforms: PLD1 and PLD2 which occur as splice variants. PLD1 is found in intracellular membranes of E.R., Golgi, and vesicular components based on fractionation studies (Colley et al., 1997; Lucocq et al., 2001), and PLD2 was found mostly in the plasma membrane (Colley et al., 1997; Park et al., 2001). It has been reported that in the mouse brain, PLD1 is expressed in neurons and PLD2 in astrocytes (Zhang, 2004). Glutamate is a potent activator of PLD in neurons but not in astrocytes (Rujano, 2004) and in expression systems PLD2 activity is required for the internalization of group I mGluRs (Bhattacharya et al., 2004). PLD appears to be linked to another subtype of mGluR that is not associated with the PLC pathway, but the specific subtype is still unknown (Albani-Torregrossa et al., 1999). It has been shown that PLD is activated by selective mGluR agonists, ACPD and DHPG, in cerebrocortical synaptosomes; this activation was not affected by a PKC inhibitor suggesting that the mGluR-linked PLD activation is PKC independent (Shinomura et al., 2000). Furthermore, a group II agonist-induced long term depression (LTD) was blocked by phospholipase D-linked mGluR blocker PCCG-13 (2R,1'S,2'R,3'S)-2-(2'Carboxy-3'-phenylcyclopropyl) glycine (Otani, 2002).

Phosphatidic acid produced by PLD as a result of signaling activity is also thought to play a role in membrane vesicle trafficking, either as an intracellular messenger or as a cone-shaped lipid that promotes membrane fusion (Roth, 1999; Liscovitch, 2000; Jones, 1999). In rat brain synaptosomes subjected to hypotonic lysis and centrifugation, PLD1 is associated with the particulate fraction containing the plasma membrane and immunostaining of rat cerebellar granule cells confirmed localization of PLD1 at the neuronal plasma membrane in zones specialized for neurotransmitter release (axonal neuritis, varicosities, and growth cone-like structures) (Humeau, 2001). Furthermore, inhibition of PLD1 blocked acetylcholine release by reducing the number of active presynaptic-releasing sites in Aplysia neurons (Humeau, 2001).

Dopamine receptors have also been shown to have a regulatory effect on PLD expression and activity. In lactotroph cells (GH4-121), D2-like receptor agonist, bromocriptine, was found to stimulate PLD activity without activation of Phospholipase

C (PLC), whereas, D2-like antagonists blocked PLD activation (Senogles, 2000). Furthermore, in HEK 293 cells, D2-like agonists specifically stimulate PLD2 (Senogles, 2003). Additionally, the D3 agonist (+)7-OH DPAT increased PLD activity in HEK 293 cells, which was blocked by D3 antagonists (Everett and Senogles, 2004). These effects were not mediated by G_i/G_o receptors because they were pertussis toxin insensitive.

Dopamine receptors and mGluRs appear to be separate signaling systems within the brain, while PLD is a downstream signaling molecule seen in many systems, but based on previous studies showing that a dopamine-induced LTP is dependent upon mGluRs (Schotanus and Cherugi, 2008), and that PLD has also been shown to interact with DRs in multiple systems (listed above), we investigated the possible interaction of these three receptors to mediate LTP in the BLA-CeA pathway after chronic cocaine.

MATERIALS AND METHODS

Animals

Male albino Sprague-Dawley rats (Harlan Houston, TX), aged 3-4 weeks at the beginning of the study were used as subjects. Animals were housed 4 per cage in a temperature and humidity controlled environment under a 12-h light/dark cycle (lights on 0700h). Food and water were available *ad libitum*. Rats were acclimated to the environment for at least 3 days before the start of an experiment. All experiments were carried out with the approval of the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Conditioned Place Preference

The conditioned place preference (CPP) behavioral paradigm has 3 stages including baseline, conditioning, and testing. The baseline and testing sessions are performed only once, while the conditioning sessions occur over 5 days. The chambers have a white side consisting of white walls and a white floor. The floorboard itself is grooved with straight rows. The black side has black walls and a black floor that is smooth. Baseline recordings last for 30 minutes. The animals are placed in an interior chamber between the black and white sides that is removed at the start of the experiment so that the rats can move freely between the white and black sides of the chamber. The experiment records the amount of time the animal spends on each side during the 30 minutes. Conditioning experiments are also 30 minutes long in which saline or cocaine injection is paired with either the black or white sides. In the morning, all animals receive saline injections. In the 4 chambers, 2 animals are placed on the black side and the other two on the white side. In the afternoon, saline animals still receive saline injections, but the cocaine animals receive cocaine injections. The animals placed on the white side in the morning were positioned on the black side in the afternoon and ones on the black side in the morning were on placed in the white in the afternoon in a counterbalanced fashion. In the afternoon, all animals also receive sound and light cues for the first 5 minutes of the session. The sound is a tone that beeps once a second for 5

minutes, simultaneously, a light flashes on for 15 seconds and off for 15 seconds. The testing session takes place on day 6, which is the day after animals receive their last injection, and is also 30 minutes. The animals were allowed to roam freely between the two chambers, as in baseline, but the testing session also includes the light and sound cues that were paired with saline or cocaine injections in the afternoons.

Electrophysiology

Animals are decapitated with a small animal guillotine. Anesthetics are not used prior to this procedure because they may interfere with and mask the neuroadaptations due to cocaine treatment and thus may confound the data. Coronal brain slices (500 μm) were prepared and placed in ice-cold (0-6º C) aerated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) composed of (all in mM) NaCl, 119; KCl 3.0; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose 11.5. Brain slices were kept at room temperature for 2 hours and then submerged in a tissue bath at 34º C $\pm$ 1º C where they were allowed to equilibrate for 30 minutes. A pH of 7.4 was maintained by continuous perfusion of ACSF (2ml/min) oxygenated with carbogen (95% O_2/5% CO_2). Recordings were performed in the pathway from the basolateral (BLA) to the central amygdala (CeA). A concentric bipolar stimulating electrode was placed in the BLA, stimulating at a frequency of 0.05 Hz, and a tungsten recording electrode was placed in the CeA. All recordings were performed in the presence of either 0 uM, 10 μM, or 50 μM picrotoxin (PTX).

Westerns Blotting

Rats were decapitated, the amygdala removed and placed on ice in a cold tris buffered saline (TBS) containing, Petri dish. The amygdala was homogenized with lysis buffer and protease inhibitor cocktail (lysis buffer from Sigma Aldrich [St. Louis, MO]). After centrifugation, the supernatant was discarded and the pellet was retained. A solution of 2X SDS (20 ul) buffer with dithiothreitol (DTT) was added and the pellet vortexed. The samples were placed in a 95° water bath for 5 minute followed by a 5 minute cooling on ice. Samples (30 μl) were separated on a gel (of 7%, 10%, and 4-12% gradients) by electrophoresis and transferred to a PVDF membrane overnight in a cold

room. Subsequently, the membrane was blocked for at least one hour in 6% milk in TBS containing 0.05% Tween-20 to reduce non-specific background staining. Primary antibodies were applied overnight at varying concentrations, also in 6% milk in TBS containing 0.05% Tween-20. The primary antibody was removed and the blot was washed with the 0.05% Tween-TBS (TTBS) 5 times for 10 minutes each. The secondary antibody was applied for 1 hour at varying concentrations depending on the antibody. Application of the secondary antibody was followed by another 5 washes with 0.05% TTBS for 10 minutes. The blot was then washed for 10 minutes with 0.05M Tris-HCL buffer. An Enhanced Chemiluminescence (ECL) kit from VWR International Incorporated (West Chester, PA) was used to develop the blots.

The primary antibodies used included: (1) rabbit polyclonal anti-mGluR1-extracellular from Alamone labs raised against the epitope peptide (C)HEGVLNIDDYKIQMNK, corresponding to amino acids 501-516 of rat mGluR1; (2) rabbit polyclonal anti-PC-PLD1 (H-160) from Santa Cruz Biotechnologies Inc. raised against amino acids 1-160 of PC-PLD1 of human origin; (3) goat polyclonal anti-PC-PLD2 (M-20) from Santa Cruz Biotechnologies Inc. raised against a peptide mapping near the N-terminus of PC-PLD2 of mouse origin; (4) and goat polyclonal anti-Actin (I-19) from Santa Cruz Biotechnologies Inc. raised against a peptide mapping at the C-terminus of Actin of human origin, varying concentrations of each were used. Secondary antibodies used included: donkey anti-rabbit from Jackson ImmunoResearch Laboratories and donkey anti-goat antibodies from Santa Cruz Biotechnologies Inc.

We also used the Licor Odyssey system to analyze western blots. The protein is obtained in the same way as described above. After protein extraction, 20 ul of 2X buffer containing DTT was added to the pellet and the protein samples were placed in a 37° water bath for 30 minutes. Next, the samples were placed on ice for 5 minutes. Samples (30 µl) were separated on a 10% gel by electrophoresis and transferred to a nitrocellulose membrane overnight in a cold room. After transfer, the blot is blocked in Licor blocking solution for at least 1 hour. Primary antibodies are applied at varying concentrations in blocking solution on a shaker overnight in a cold room. Blots are washed 4 times 5

minutes with PBS containing 0.5% Tween. Secondary antibodies are applied in blocking solution for 1 hour. Blot is then washed 4 times 5 minutes with PBS-T and once for 5 minutes with PBS. The blot is visualized using the Licor Odyssey System.

Fractionation

The protein in each sample is completely homogenized with 1 ml of lysis buffer containing 1% Triton X-100 in a glass homogenizing tube. The samples are returned to eppendorf tubes, allowed to incubate on ice for 10 minutes and then spun down in the cold room at 3000 rpm for 15 minutes. The entire supernatant is removed without disturbing the pellet and transferred to small, thin centrifuge tubes. The pellet is set aside and saved. All samples are weighed and balanced in pairs and then centrifuged at 38200 rpm for 30 minutes at 4°C. Samples are separated into supernatant and pellet fractions are stored at -80°C.

Co-immunoprecipitation

Animals were decapitated and the protein from the amygdala was collected in the same manner as for western blotting. For each sample, 150 µg of the whole amygdala extract was used. The desired antibody (5-10 µg) was added to each protein sample and was incubated overnight in the cold room (4°C) on a rotating shaker at a moderate speed. The next day, 20 µl of 50% diluted Pierce Protein A/G beads were added to each protein sample and placed back on the shaker 4°C overnight. Before adding the beads to the protein samples, the beads were washed 3 times with 100 µl lysis buffer containing 1% Triton X-100. In between washes, the beads are centrifuged and the supernatant in discarded. After the beads are slurried, they are immediately added to protein samples. The next day, samples were centrifuged at 14,000 rpm for 5 minutes in the cold room. The supernatant was removed and the samples were washed 2 times with 100 µl of lysis buffer containing 1% Triton X-100 and one wash with 100 µl lysis buffer containing 0.25% Triton X-100. Samples were centrifuged for 30 seconds between washes and the pellet was discarded. The pellet and about 10 µl of the supernatant were saved and 20 µL of 2X SDS Buffer with DTT were added to each sample. Further steps were performed as described above for westerns.

PLD Activity Assay

Animals were decapitated without anesthesia and 350 μm coronal brain slices were collected and placed in ice-cold (0-6º C) aerated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) composed of NaCl, 119 mM; KCl 3.0 mM; NaH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM; and glucose 11.5 mM. The amygdala was dissected out from each brain slice and placed in test tubes containing about 2 ml of Kreb's Bicarbonate Buffer consisting of NaCl 22 mM; KCl 3.1 mM; $MgSO_4$ 1.2 mM; KH_2PO_4 0.4 mM; and $CaCl_2$ 1.3 mM. The pH of the buffer containing the slices was maintained at 7.4 throughout the day by continuous addition of carbogen (95% O_2/5% CO_2). The slices remained in the test tubes at room temperature for 30 minutes, then were placed in a water bath (37°C) for 30 additional minutes. The Kreb's buffer was removed from the test tubes and replaced by 1 ml of ACSF containing 30 μCi of tritiated glycerol per sample tested. The slices were exposed to the ACSF containing the radioactive material for 2 hours. The slices were then washed with 2 ml of ACSF and replaced by 500 μl of ACSF, containing only ethanol (5 ul per sample), ethanol and a drug, or an additional ACSF equivalent for a negative control. The slices remain exposed to the drug and ethanol for 1 hour while remaining in the water bath. The reaction was stopped by addition of 2 mL of ice-cold chloroform/methanol/HCl (100:200:2) to each test tube. The samples were sonicated for 30 minutes and then centrifuged at 3000 rpm for 2 minutes. Two layers formed and 1 ml of the lower organic layer was removed from each tube and pipetted into a separate test tube. The organic layers were dried by N2 gas and 70 μl of chloroform were added to the dried substance remaining in each tube. The 70 μl of chloroform from each tube were spotted on a silica gel coated thin layer chromatography (TLC) plate in about 3 μl increments. The plate was placed in a large rectangular glass chamber containing a minimum of 100 mL of a solvent system consisting of ethyl acetate: 2,2,4 – trimethyl pentane (also called iso-octane): acetic acid: methanol: water in 60:80:20:20:10 ratio chamber. After 30 minutes, the plate was removed from the glass running chamber and placed in a chamber containing a layer of iodine crystals on the bottom for 15 minutes. The phosphatidylethanol (PEtOH) sample

was determined by collecting 1 mm of sample from the plate in between the PA and PEtOH standards. The phospholipids remaining in the lanes of each sample were scraped from the plate and collected in individual vials of scintillation fluid to determine the ratio of PEt/Total lipids.

Injections

Cannula were placed in the amygdala of rats when approximately three weeks of age. The animals recovered for five days after surgery and then began CPP, starting with baseline as usual. After two weeks withdrawal, animals were injected with the drug, PCCG-13, 15 minutes before the test session.

Drugs

The National Institute of Drug Abuse provided the cocaine HCl. Cocaine (15 mg/ml) was dissolved in 0.9% saline solution and injected intraperitoneally (i.p.) at a concentration of 15mg/kg. PTX was purchased from Sigma Aldrich (St. Louis, MO). Dopamine and raclopride were also purchased from Sigma Aldrich and were dissolved in water. MPEP HCl was obtained from Ascent Scientific (North Somerset,United Kingdom). SKF81297, LY367385, PCCG-13, L-CSA, U-73122, and SCH23390 were obtained from Tocris Bioscience (Ellisville, MO). For *in vitro* experiments, drugs were superfused in ACSF at their final concentrations.

RESULTS

Conditioned Place Preference (CPP) with cocaine was detected on both black and white sides of the chamber.

In previous studies in our laboratory, CPP was established using a biased design in which cocaine animals were paired with the non-preferred (white side) (data not shown). For the afternoon treatments in those experiments all saline animals received saline on the black side and all cocaine animals received cocaine injections on the white side of the chamber. Earlier in the day saline animals received saline on the white side and cocaine animals received saline on the black side. The present set of experiments shown in **Figure 1** represented a counterbalance control to test whether cocaine animals would still prefer the black side over the white side if they received cocaine injections on the black side of the chamber. Animals were allowed to roam freely between the black and white sides of the chamber for the 30 minute test session. In these experiments one half of the cocaine treatment group who, as in previous experiments, received their injections on the white side had significantly greater CPP scores than saline animals receiving saline injections on the white side (cocaine=194.0 ± 72.1, saline= -255.2 ± 72.2, $p<0.0001$, n=19),

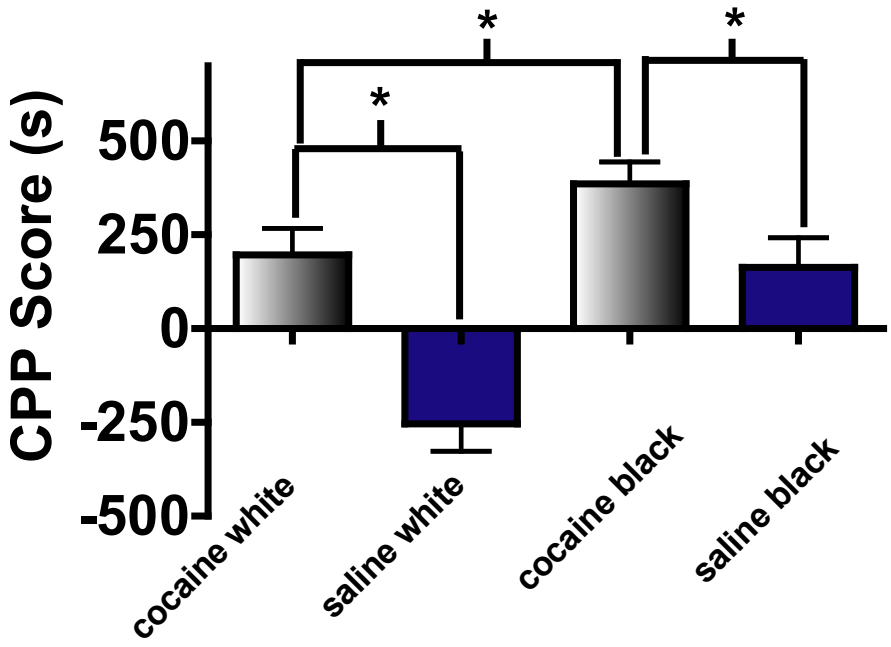

Figure 1: Counterbalanced experiments: Cocaine injected animals preferred the side paired with cocaine injections while the saline animals preferred the black side over the white regardless of saline pairing. Graph depicts CPP scores determined by time spent on drug-paired side during 30 minute test sessions minus the time spent on that side during baseline measurement. Positive values indicate that the animal chose the injection paired side and spent more time on that side during the test session than during baseline. Half of the cocaine animals had their cocaine injections paired with the black side while the other half was paired with the white side. A similar paradigm was run with saline animals. The values for the black side are all significantly different from the mean indicating a preference by each group (n=19 for each group) $p<0.001$*.

showing that cocaine-treated animals spent more time on the white side on their test day than during baseline recordings. These data indicated that CPP occurred in cocaine animals when cocaine is paired with the non-preferred white side and that the saline animals did not develop a preference for the white side due to saline injections. The other half of the animals received cocaine injections on the black side of the chamber; their CPP scores were also positive and significantly different from animals receiving cocaine on the white side (cocaine white= 194.0 ± 72.1, cocaine black= 384.5, ± 58.8, $p=0.04$, n=19), which suggested that the animals preferred the drug paired side on test day 6 irrespective of the side of chamber paired with the drug.

SKF81297-induced LTP in the basolateral to central amygdala pathway in the cocaine- but not saline-treated group.

Cocaine-seeking behavior and extracellular dopamine levels in the amygdala increase after cocaine withdrawal and dopamine receptor agonists reinstate cocaine-seeking behavior (Tran-Nguyen et al., 1998). SKF81297, a selective D1 agonist, was applied to determine if activating dopamine receptors could affect synaptic plasticity in slices from cocaine and saline animals. To characterize dopamine receptor activation during cocaine withdrawal, we analyzed the effects of SKF81297 (10μM), on field excitatory postsynaptic potentials (fEPSP) in the BLA to CeA pathway 14 days after the last cocaine or saline injection.

SKF81297 induced LTP (151.4% ± 8.8 %, t=5.87, p=0.002, n=6) of fEPSPs in slices from cocaine animals **(Figure 2A)** whereas saline fEPSP magnitude did not differ significantly from the baseline (101.6% ± 9.7, t=0.16, n=7), indicating that SKF81297 was unable to induce LTP in saline slices. fEPSPs in slices from the cocaine group were significantly different from saline values (t=4.74, n=6), suggesting a difference in SKF81297 effect in the cocaine compared to saline group. These data suggested that during cocaine withdrawal SKF81297-induced LTP was enhanced in the amygdala.

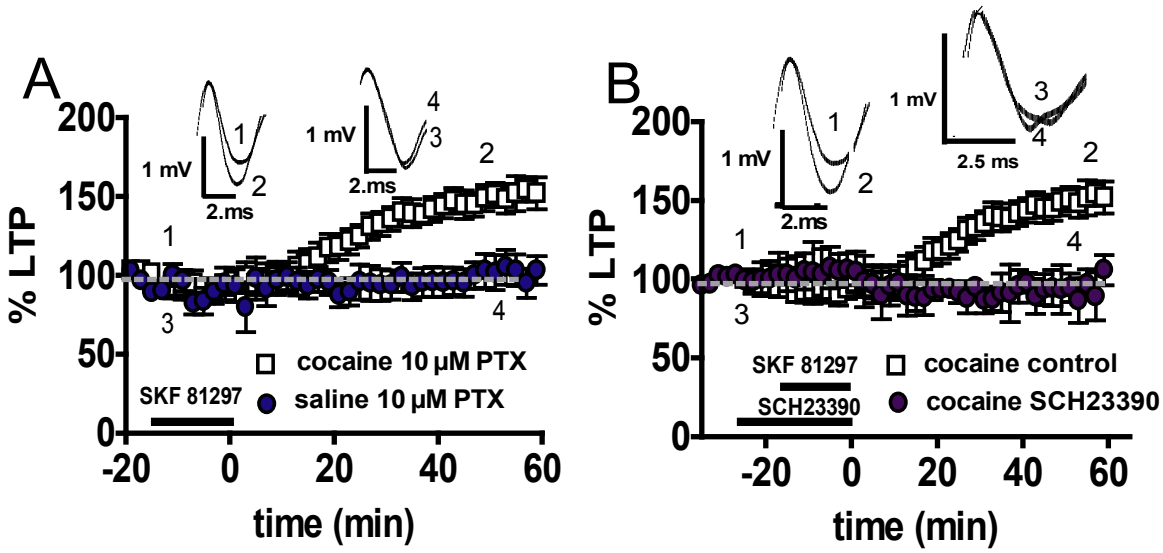

Figure 2: SKF81297-induced LTP in the BLA to CeA pathway in slices from cocaine, but not saline treated animals. The change in fEPSP slope (% of baseline) is plotted as a function over time (min). Baseline fEPSPs were recorded for 10 min and 10 µM SKF81297 applied for 15 minutes followed by a post-drug recording of 60 minutes. In the SCH23390 recordings, baseline was followed by 10 minutes of 10 µM SCH23390 and the combination of 10 µM SKF81297 with 10 µM SCH23390 applied for 15 minutes followed by a post-drug recording of 60 minutes. All recordings were performed in the presence of 10 µM PTX.

To ensure that the LTP recorded in cocaine slices from cocaine treated animals in **Figure 2A** was indeed due to application of SKF81297, we tested the effects of the D1 antagonist, SCH23390, on the SKF81297-induced LTP **(Figure 2B)**. The SKF81297-induced LTP in the presence of SCH23390 was significantly reduced compared to the SKF81297 control ($t=6.35$, $p=0.008$, $n=4$).

In order to determine whether the concentration of SKF81297 induced a maximal response, we also tested 25 µM SKF81297 on the SKF-induced LTP in the presence of 10 µM PTX. The LTP recorded in the presence of this increased SKF concentration in slices from cocaine-treated animals (135.5% ± 5.9) was significantly different from the baseline ($t=6.07$, $n=3$, $p=0.009$) and its saline ($t=4.84$, $n=3$, $p=0.017$) counterpart **(Figure 3)**. This LTP, however, was not significantly different from the 10 µM SKF81297-

induced LTP (t=0.324, n=3, p=0.767) or from the dopamine-induced LTP (t=1.20, n=3, p=0.317). We did not investigate the effects of a concentration lower than 10 µM. Since the lower SKF81297 concentration, 10µM, induced a maximal response, we use that concentration in subsequent experiments.

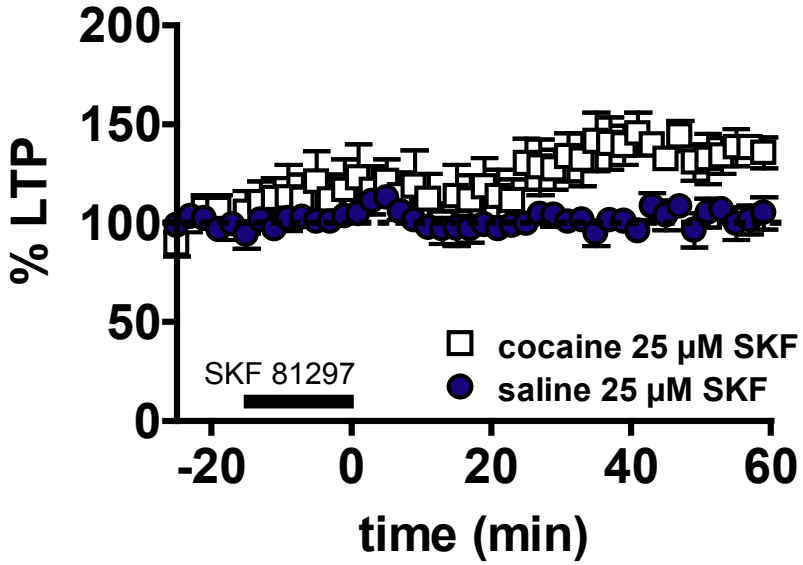

Figure 3: SKF81297-induced LTP still seen in 25 µM SKF81297. The change in fEPSP slope (% of baseline) is plotted as a function over time (min). Baseline fEPSPs were recorded for 10 min and 25 µM SKF81297 applied for 15 minutes followed by a post-drug recording of 60 minutes. All recordings were performed in the presence of 10 µM PTX.

Effect of dopamine also induced LTP in the BLA-CeA pathway.

To determine if the effects of SKF81297 are mimicked by the endogenous neurotransmitter, dopamine, we applied exogenous dopamine in the presence of the D2 receptor antagonist, raclopride (**Figure 4A**). The dopamine/raclopride-induced LTP was significantly greater than the baseline values (146.5 ± 3.2, t=14.7, n=5), and significantly different from values recorded in saline control slices (cocaine: 146.5 ± 3.2; saline: 102.2 ± 2.4, t=12.9, n=5, *p<0.05).

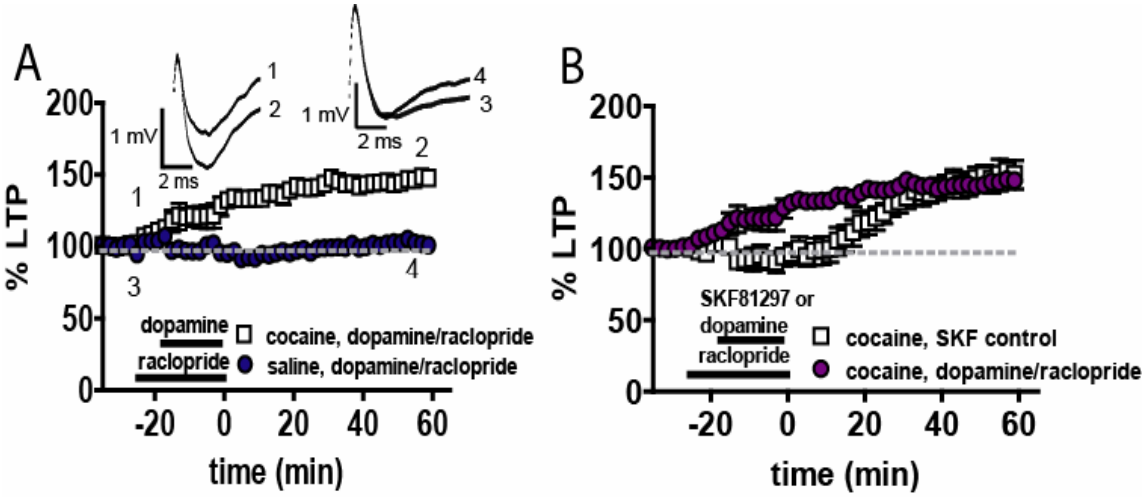

Figure 4: Dopamine-induced LTP in the presence of raclopride mimicked the SKF81297-induced LTP in cocaine slices at the BLA-CeA synapse. After baseline, 10 μM raclopride was applied for 10 minutes, followed by a combination of 10 μM raclopride and 10 μM dopamine applied for 15 minutes and a post-drug recording of 60 minutes. Recordings were performed in 10 μM PTX.

Furthermore, although the time courses of LTP induced by the two agonists were different **(Figure 4B)**, fEPSP values for the last 10 minutes of SKF81297- or dopamine/raclopride-induced LTP were not significantly different (cocaine, dopamine: 146.5 ± 3.2; cocaine, SKF81297: 151.4 ± 8.8, t=0.22, n=5). Additionally, the time course differences between the synthetic compound and the endogenous neurotransmitter were consistent between multiple recordings.

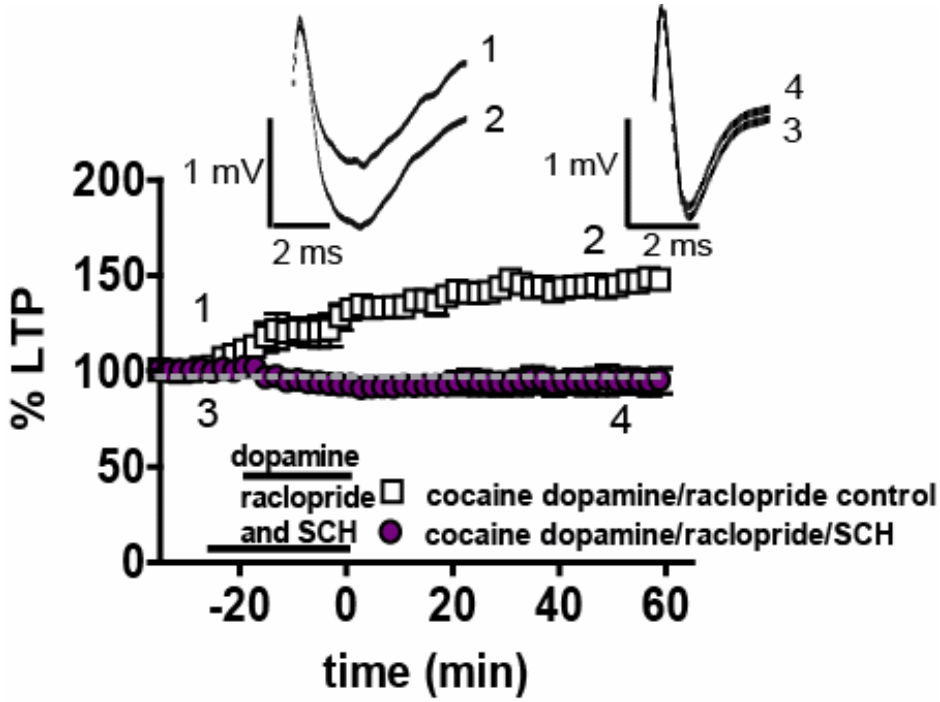

Figure 5: Dopamine/raclopride-induced LTP was blocked by the D1 antagonist, SCH23390, in the BLA-CeA pathway in slices from cocaine withdrawn animals. After baseline, 10 μM raclopride was applied for 10 minutes, followed by a combination of 10 μM raclopride, 10 μM SCH23390, and 10 μM dopamine applied for 15 minutes and a post-drug recording of 60 minutes. Recordings were performed in 10 μM PTX.

SCH23390 blocked the dopamine-induced LTP in the BLA-CeA pathway.

The ability of dopamine to induce LTP in the presence of the D2-like antagonist, raclopride, indicated that the dopamine-induced LTP in the cocaine group was mediated through D1-like receptors, D1 or D5. The fact that that the dopamine-induced LTP mimicked the SKF81297-induced LTP suggested that it was mediated through D1 and not D5 receptors. We verified this data by testing the effect of a D1 antagonist (**Figure 5**), and showed that the dopamine-induced LTP, in the presence of raclopride, was completely blocked by the D1 antagonist, SCH23390, compared to the dopamine/raclopride control ($t=7.87$, $n=5$, $p=.0014$). Additionally, the reduction in dopamine-induced LTP due to SCH23390 was also significantly reduced compared to the SK81297 control ($t=12.33$, $n=6$, $p=.0001$ data not shown).

Presence of gamma-aminobutyric acid (GABA) inhibition was required for SKF81297-induced LTP.

Dopamine receptors are located on interneurons in the amygdala (Marowsky et al., 2005). To determine if the SKF81297-induced LTP was dependent on GABAergic inhibition, we analyzed the concentration-response effects of the $GABA_A$ receptor non-competitive antagonist, picrotoxin (PTX). We next tested the concentration-response relationship for PTX (0 μM, 10 μM, and 50 μM PTX; **Figure 6**). The data showed dependence of the SKF81297-induced LTP on the presence of synaptic inhibition. When the PTX concentration was increased to 50 μM in saline slices, the fEPSP magnitude did not differ significantly from baseline values (101.1 ± 5.1, t=0.16, n=7; p>0.05) **(Figure 6C)**. The differences in LTP magnitude between cocaine and saline treated groups measured in 10 μM PTX were also abolished in the presence of 50 μM PTX (10μM: 112.2 ± 4.6; 50μM: 101.1 ± 5.1, t=1.66, n=7; p>0.05) **(Figure 6C)**. However, in 50μM PTX, the fEPSP magnitude was significantly different from the baseline (112.2 ± 4.6, t=2.63, p<0.039, n=7), suggesting that a slight SKF81297-induced LTP remained in the cocaine group. Although SKF821297 induced LTP in 50 μM PTX, it was significantly reduced compared to the SKF81297-induced LTP recorded in the presence of 10 μM PTX (50μM: 112.2 ± 4.6; 10μM: 151.4 ± 8.8, t=5.87, n=6; **Figure 6B)** in the cocaine group. These data suggested that saline recordings were unaffected by 50 μM PTX and that the effects of dopamine agonists in inducing LTP was dependent on the presence of GABA transmission since blocking inhibition with increased PTX concentration blocked the effects of the D1 agonist, SKF81297.

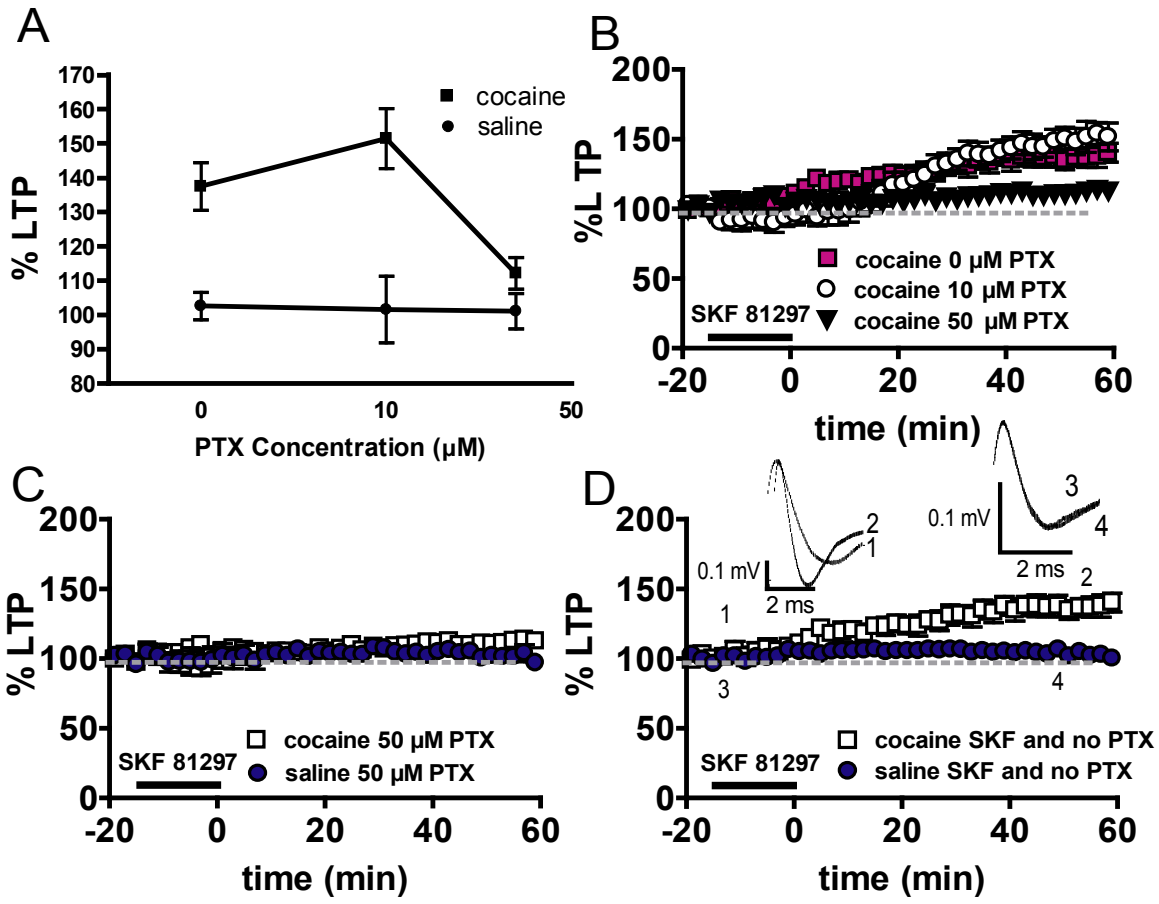

Figure 6: SKF81297-induced LTP in cocaine recordings in the absence of picrotoxin but an increase in PTX concentration (50 μM) blocked SKF81297-induced LTP in the BLA-CeA pathway with no effect on SKF recordings in slices from saline-treated animals. Recordings were performed in 50 μM or 0 μM picrotoxin and compared to those in 10 μM PTX. Panel A is a concentration-response curve for PTX. Panel B shows the time course of the LTP in cocaine for all three PTX concentrations. Panel C shows the comparison of LTP in the presence of 50 μM PTX in slices from cocaine- and saline-treated animals. Panel D shows the comparison of LTP in the absence of PTX in slices from cocaine- and saline-treated animals.

An important question was whether D1-mediated LTP can occur without modification of inhibition, a state that may more closely mimic the "native" control state of the neurons. fEPSP magnitudes in slices from cocaine-treated animals were significantly different from the baseline (137.5 ± 6.9, $t=5.41$, $p<0.05$, $n=5$**; Figure 6D**). These values were also significantly greater than those in saline slices, which were not

significantly different from the baseline (102.6 ± 4.03, t=0.64, n=5). SKF81297-induced LTP in cocaine slices in the absence of picrotoxin was significantly greater than that in the presence of 50 μM picrotoxin (0 μM PTX: 137.5 ± 6.9; 50 μM PTX: 112.2 ± 4.63, t=3.02, n=5; $p<0.05$). fEPSP values for the cocaine-treated group in 50 μM PTX were also significantly less than those in 10 μM PTX LTP obtained in 0μM and 10 μM PTX were not significantly different from each other (10μM: 151.4 ± 8.8; 0μM: 137.5 ± 6.9, t=1.40, n=5; $p>0.05$; **Figure 6B**). These data clearly indicated that some intact inhibition was required for the SKF81297-induced LTP in the BLA-CeA pathway in cocaine slices. Additionally, fEPSP values in cocaine slices in 0μM and 10 μM PTX were significantly increased compared to those obtained in the presence of 50 μM PTX indicating a significant effect of different PTX concentrations on SKF81297-induced LTP in the BLA-CeA pathway and when analyzed with a one-way ANOVA, the three groups were significantly different ($p=0.011$). These data indicated that the SKF81297-induced synaptic potentiation in cocaine slices required the presence of some synaptic inhibition. Additionally, these data suggested that the SKF81297-induced LTP at the BLA-CeA synapse was interneuron dependent. Based on these results, we used the 10 μM concentration of PTX for all subsequent studies due to the fact that the LTP seen in the presence of 10 μM PTX was not significantly greater than the LTP in the absence of PTX, but it was slightly more robust than in the absence of PTX. Thus we chose to use the lowest concentration of PTX which also had the most robust effect on the SKF-induced LTP.

The SKF81297-induced LTP was linked to a metabotropic glutamate receptor induced LTP.

Activation of D2-like dopamine receptors is thought to be linked to PLD (Yang, Z et al., 2005; Senogles, 2000; Senogles, 2003, Senogles, 2007). Furthermore, dopamine receptors are known to regulate the signaling properties of group I metabotropic glutamate receptors (Poisik et al., 2007). Since specific inhibitors of PLD were not

available we examined the possible interaction between dopamine receptors and PLD by analyzing

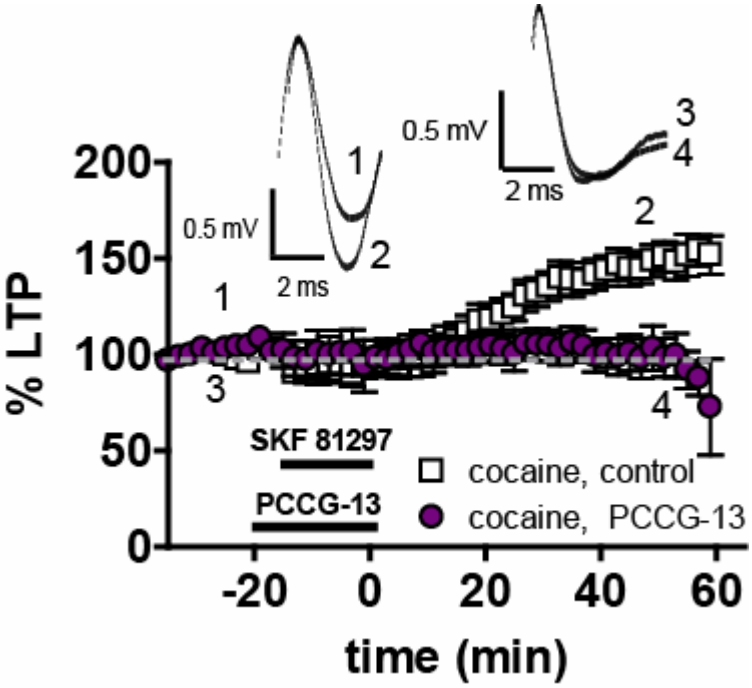

Figure 7: PCCG-13 (2 μM) blocked SKF81297-induced LTP in the cocaine-treated group. After baseline recording, 2 μM PCCG-13 was applied for 10 minutes, followed by a combination of 2 μM PCCG-13 and 10 μM SKF81297 applied for 15 minutes and a post-drug recording of 60 minutes. Entire duration of recording was also performed in the presence of 10 μM PTX.

the effect of a specific antagonist, (2*R*,1'*S*,2'*R*,3'*S*)-2-(2'-carboxy-3'-phenylcyclopropyl) glycine (PCCG-13) stereoisomer, which antagonizes PLD activity by blocking the mGluR-linked to PLD (Albani-Torregrossa et al., 1999). In the presence of PCCG-13, fEPSP magnitudes in the cocaine-treated group were not significantly different from the baseline (95.0 ± 9.2 %; t=0.55, p<0.05, n=6; **Figure 7**). Importantly, the SKF-induced LTP was significantly different than that recorded in the presence of 2 μM PCCG-13 (cocaine, control: 151.4 ± 8.8; cocaine, PCCG: 95.0 ± 9.2, t=3.40, n=6). These data suggested that the SKF81297-induced LTP in slices from cocaine-treated animals was dependent on PLD activation since it was prevented when the mGluR linked to PLD was blocked. fEPSPs in saline slices were not significantly different from the baseline (104.3

± 8.2%, t=0.525, n=6), indicating that PCCG-13 did not affect saline control slices (**Figure 8A**).

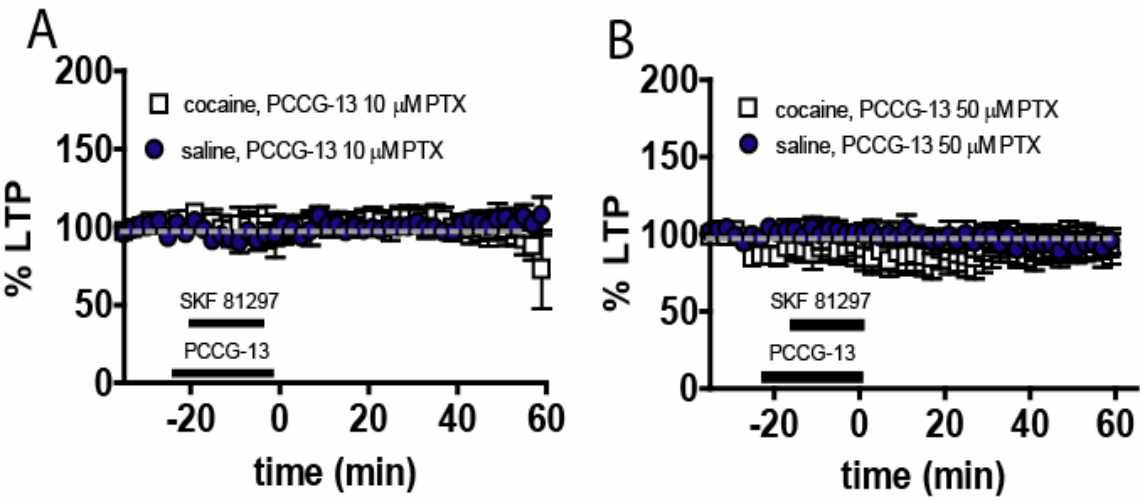

Figure 8: PCCG-13 blocked SKF induced-LTP in cocaine slices in 10 μM (A) as well as 50 μM (B) PTX. PCCG-13 (2 μM) was applied for 10 minutes and a combination of 2 μM PCCG-13 and 10 μM SKF81297 was applied for an additional 15 minutes followed by a post-drug recording of 60 minutes. fEPSPs recorded in the presence of 2 μM PCCG-13 were compared in the presence of 10 μM (panel A) and 50 μM (panel B) PTX in slices from cocaine and saline-treated animals.

Additionally, activity induction in the presence of 2 μM PCCG-13, 10 μM SKF81297, and 50 μM PTX in the cocaine-treated population was not significantly different from the baseline (93.7 ± 10.1, t=0.627, n=5; **Figure 8B**). Saline fEPSP magnitudes in PCCG-13 with50 μM PTX were also not significantly different from the baseline (92.7 ± 9.7, t=0.748, n=5) and from those in the presence of SKF81297, PCCG-13, and 10 μM PTX ($p>0.05$). These results indicated that the SKF81297-induced LTP was blocked by PCCG-13 in the cocaine group, while the saline group was unaffected. This suggested that the D1-mediated LTP in the BLA-CeA pathway may be dependent upon the specific mGluR-linked to PLD.

SKF81297-induced LTP was mediated through an mGluR1 receptor.

The block of the SKF81297-induced LTP by the PCCG-13, a selective antagonist of the mGluR-linked to PLD, suggested the D_1 agonist-induced LTP was mediated

through an mGluR. To examine the mGluR subtype linked to PLD we tested the effect of LY367385, a competitive mGluR1 antagonist. LTP values (**Fig 9A)** in 100 µM LY367385 were significantly different from each other in cocaine group (cocaine, 10 µM PTX: 151.4 ± 8.8; cocaine, PTX 10 µM, 100 µM LY367385: 106.0 ± 6.7, t=3.62, n=6, $p<0.05$). These data suggested a role for mGluR1 receptors in the D1-mediated LTP. It has been shown recently that D1, but not D2 receptors work with glutamate receptors to induce LTP in the nucleus accumbens (Schotanus and Cherugi, 2008)

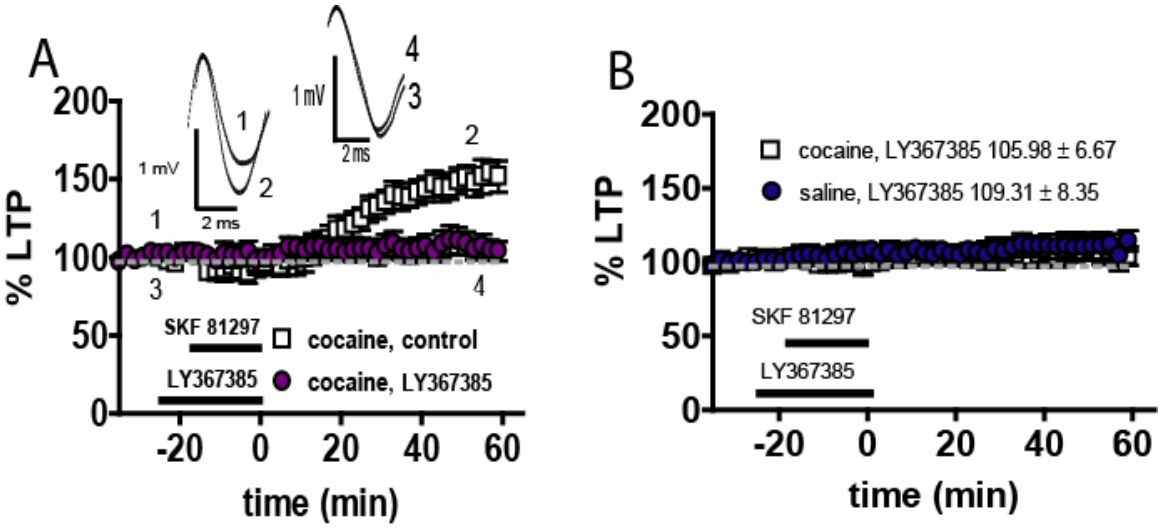

Figure 9: A selective competitive mGluR1 antagonist blocks D1 agonist-induced LTP. Graph depicts the change in fEPSP slope (% of baseline) over time (min). After baseline 100 µM LY367385 was applied for 10 minutes, followed by the combination of 100 µM LY367385 and 10 µM SKF81297 applied for 15 minutes and a post-drug recording of 60 minutes. PTX (10 µM) was present throughout the recordings. Panel A depicts the comparison of the SKF81297 cocaine control recording compared to SKF81297 administration in the presence of LY367385 in slices from cocaine-treated animals. Panel B depicts the comparison of SKF81297 in the presence of LY367385 in slices from cocaine- and saline-treated animals.

SKF-induced LTP in the presence of 100µM LY367385 did not differ significantly from the baseline (106.0 ± 6.7%, t=0.896, n=6) **(Figure 9B)**. Additionally, fEPSP magnitudes were not significantly different from the baseline in the saline controls (109.3 ± 8.4, t=1.12, n=6). In 100 µM LY367385 (saline: 109.3 ± 8.4%; cocaine: 106.0 ± 6.7%, t=0.3642, n=6), LTP magnitudes in saline and cocaine groups did not differ

significantly, indicating that the SKF81297-induced LTP in slices from cocaine-treated animals was not different from saline control levels in LY367385. These data suggested that the SKF81297-induced LTP in cocaine was dependent on an mGluR1 subtype.

SKF81297-induced LTP was also dependent on mGluR5.

Group I mGluRs, both mGluR1 and mGluR5, are known to play major roles in addiction (Swanson et al., 2001). Additionally, the mGluR5 antagonist, MPEP, was able to block the induction of LTP in the amygdala (Fendt and Schmid, 2002). In the BLA-CeA pathway, the competitive mGluR5 antagonist, MPEP, reduced the SKF-induced LTP in the cocaine group **(Figure 10A)**. SKF81297-induced LTP generated in the presence of MPEP was significantly reduced in the cocaine group compared to cocaine SKF81297 control ($t=4.11$, $n=6$, $p=0.009$), indicating that mGluR5 activation contributed to the SKF81297-induced LTP. The SKF-induced LTP in MPEP was significantly different from

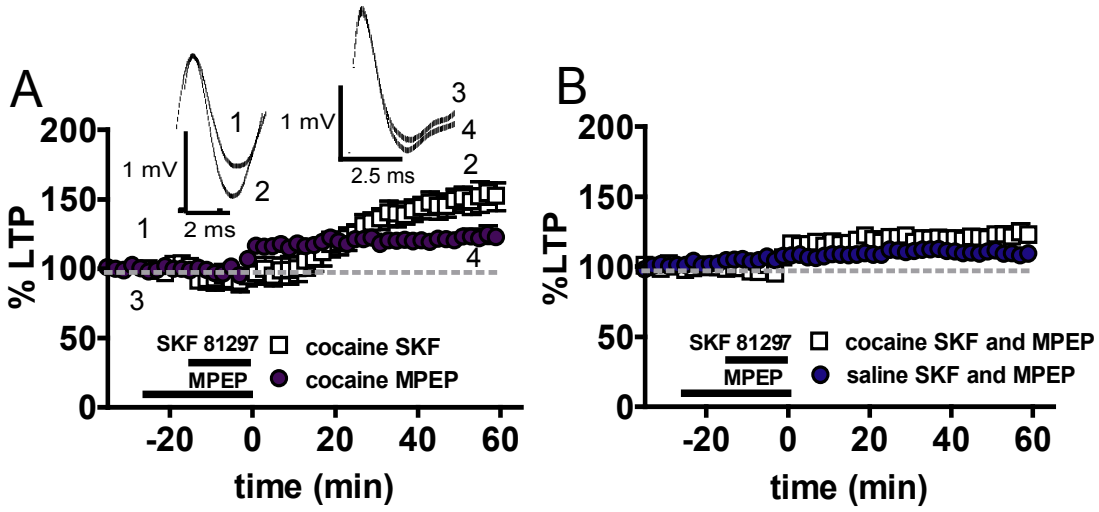

Figure 10: SKF81297-induced LTP is reduced by the mGluR5 antagonist, MPEP, compared to the SKF control in cocaine slices at the BLA-CeA synapse. After baseline recording, 10 μM MPEP was applied for 10 minutes, followed by a combination of 10 μM MPEP and 10 μM SKF81297 applied for 15 minutes and a post-drug recording for 60 minutes. All recordings were preformed in the presence of 10 μM PTX. Panel A shows the comparison the SKF control compared to SKF administration in the presence of MPEP in slices from cocaine-treated animals. Panel B depicts the comparison of SKF in the presence of MPEP in slices from cocaine- and saline-treated animals.

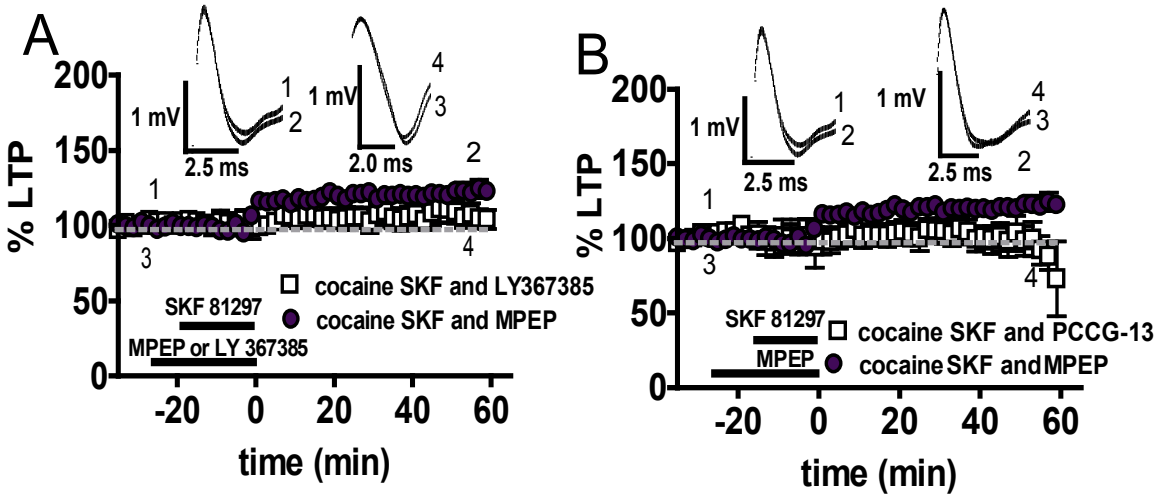

Figure 11: SKF81297-induced LTP was reduced by MPEP to a lesser extent than with LY367385 in the BLA-CeA pathway in slices from cocaine CPP animals. After baseline recording, 10 μM MPEP was applied for 10 minutes, followed by a combination of 10 μM SKF81297 and 10 μM MPEP or 10 μM LY367385 applied for 15 minutes and a post-drug recording for 60 minutes. All recordings were preformed in the presence of 10 μM PTX. Panel A compares the SKF-induced LTP in the presence of LY367385 with that in the presence of MPEP in slices form cocaine-treated animals. Panel B compares the SKF-induced LTP in the presence of PCCG-13 with that in the presence of MPEP in slices from cocaine-treated animals.

the baseline (t=4.08, n=8, p=0.005) but not significantly different from saline control **(Figure 10B)**. However, LTP magnitude in MPEP is significantly different than that in LY367385 (t=2.75, n=6, p=0.04) **(Figure 11A)** indicating that MPEP only diminished, while LY367385 completely blocked the dopaminergic LTP suggesting perhaps, a different role for the two mGluRs in mediating the SKF81297-induced LTP in the cocaine group. The SKF81297-induced LTP remaining in MPEP (**Figure 11B)** was also significantly different from SKF81297-induced LTP in PCCG-13 (t=3.237, n=6, p=0.02). Since the two mGluRs may have different signaling mechanisms contributing to

SKF81297 actions, the differences in mGluR 1 and 5 effects suggested that PLD may not linked to an mGluR5 receptor.

Inhibiting PLC attenuated the SKF81297-induced LTP in the BLA-CeA pathway.

Group I mGluRs (mGluR1 and mGluR5) are positively coupled to phospholipase C (PLC) in hippocampal CA1 neurons (Fenguelli et al. 1993) and Purkinje cells (Yukazi and Mikoshiba 1992).

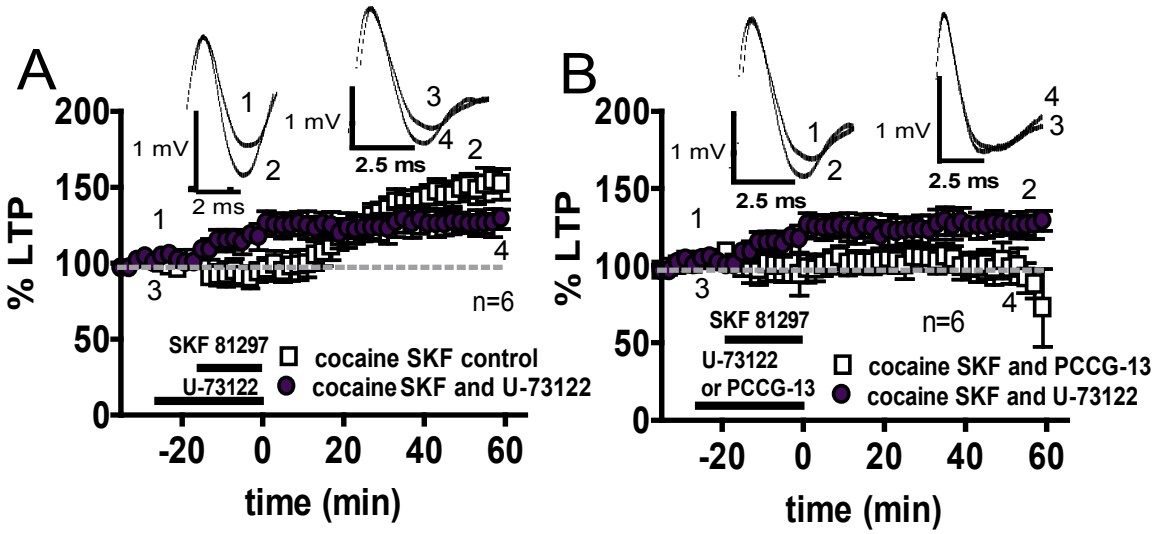

Figure 12: SKF81297-induced LTP is reduced by the PLC antagonist, U-73122 (A), in cocaine slices within the BLA-CeA synapse, but unlike PCCG-13 (B) does not block LTP. After baseline recording, 10 μM MPEP was applied for 10 minutes, followed by a combination of 10 μM MPEP and 10 μM SKF81297 applied for 15 minutes and a post-drug recording for 60 minutes. All recordings were preformed in the presence of 10 μM PTX. Panel A compares the SKF control with the LTP in the presence of SKF and U-73122 in slices from cocaine-treated animals. Panel B compares the SKF-induced LTP in the presence of PCCG-13 with that in the presence of U-73122 in slices from cocaine-treated animals.

The SKF81297-induced LTP was significantly reduced by the PLC inhibitor, U-73122, compared to the SKF81297 control ($t=5.143$, $n=6$, $p=0.004$) but was still significantly greater than the baseline ($t=4.626$, $n=6$, $p=0.006$) **(Figure 12A)**. Additionally, the reduced LTP recorded in the presence of U-73122 was still significantly greater than that measured in the presence of PCCG-13 ($t=2.846$, $n=6$, $p=0.04$) **(Figure 12B)**. These data indicated that while the antagonist for the mGluR-linked to PLD completely blocked the

LTP, the PLC antagonist only reduced this LTP suggesting pathways modulating the SKF81297-induced LTP may include both PLC and PLD.

PLD protein expression in the amygdala was increased during withdrawal in cocaine CPP trained animals.

The above electrophysiology studies demonstrated that PCCG-13, an antagonist of the mGluR-linked to PLD, blocked the SKF81297-induced LTP. We next analyzed the effects of cocaine CPP on PLD protein expression two weeks after the last cocaine injection.

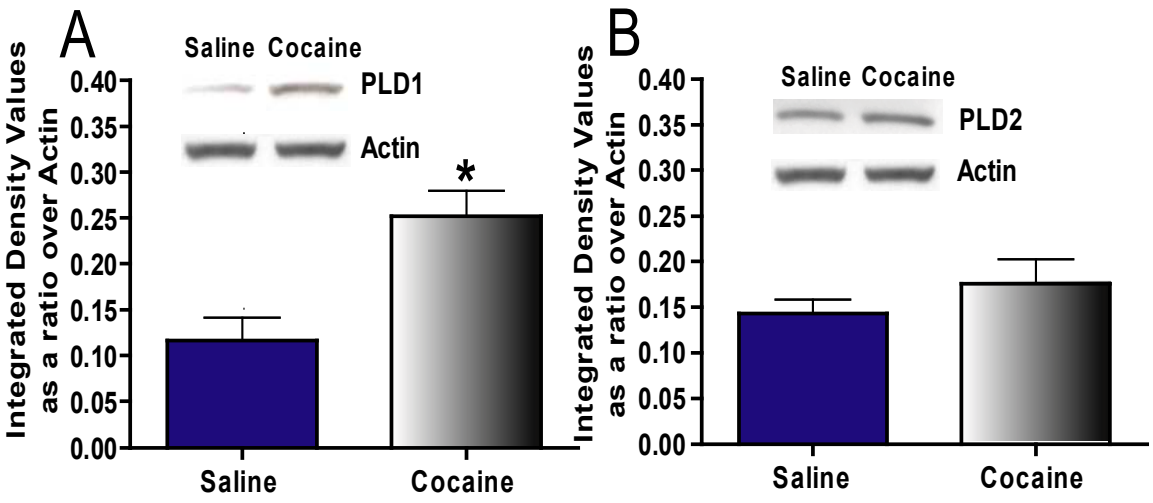

Figure 13: PLD1 expression in the amygdala was increased in cocaine- compared to saline-treated animals but PLD2 expression was not significantly changed.Graph shows changes in protein expression in cocaine- and saline-treated CPP animals. The upper band in the blots is either PLD1 (Panel A) or PLD2 (Panel B) and the lower band is actin (p=0.003).

The expected band at 120kd for PLD1 was detected, and PLD1 protein expression was significantly increased in whole cell lysate of amygdala obtained from cocaine-treated animals compared to the saline controls (t=19.9, n=3, p=0.003) **(Figure 13A)**. However, no significant difference in PLD2 protein expression was detected in cocaine- and saline-treated animals (t=1.27, n=4, p=0.29) for the expected 120 kd band **(Figure 13B)**. These

data suggested that PLD1, not PLD2 was the PLD subtype linked to the mGluR mediating SKF8129-induced LTP. Since we did not detect a change in PLD2 receptor levels in whole amygdala lysate, we analyzed whether PLD1 and PLD2 protein could be detected in the membrane fraction. PLD1 expression in the amygdala in membrane fractions from cocaine-treated animals was significantly increased (t=2.20, n=3, p=0.04, one-tailed test**; Figure 14A)**. We also measured a significant increase in PLD2 receptor protein in the membrane fraction in cocaine-compared to saline-treated animals (t=2.23, n=3, p=0.04, one-tailed test) **(Figure 14B)**. Both PLD1 and PLD2 bands were visualized at their expected molecular weight of 120 kd.

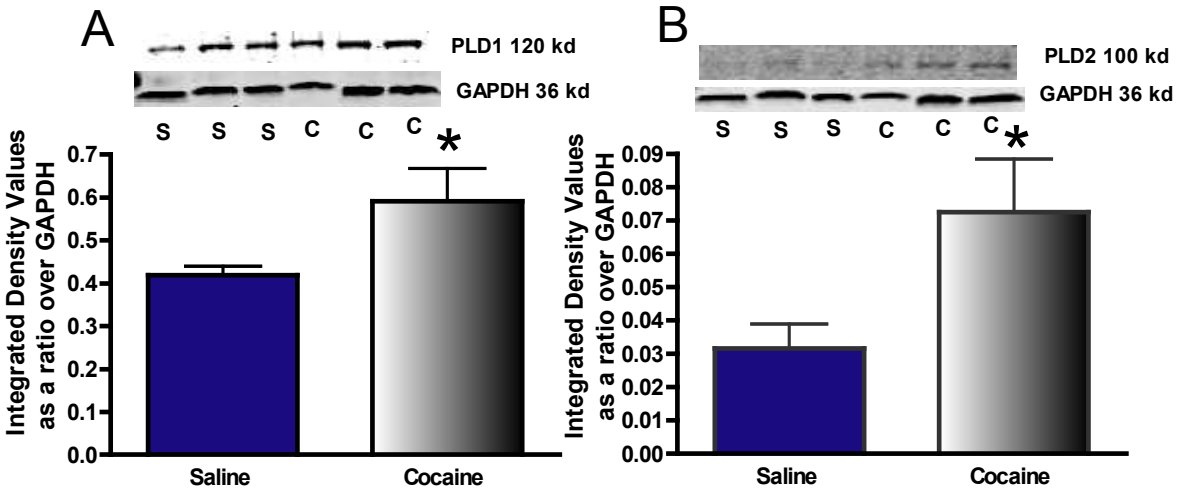

Figure 14: PLD1 and PLD2 protein expression was significantly increased in the membrane fraction of the amygdala from cocaine-treated animals. Graph shows protein expression changes in cocaine- and saline-treated CPP animals. The upper band in the blots is either PLD1 (Panel A) or PLD2 (Panel B) and the lower band is GAPDH.

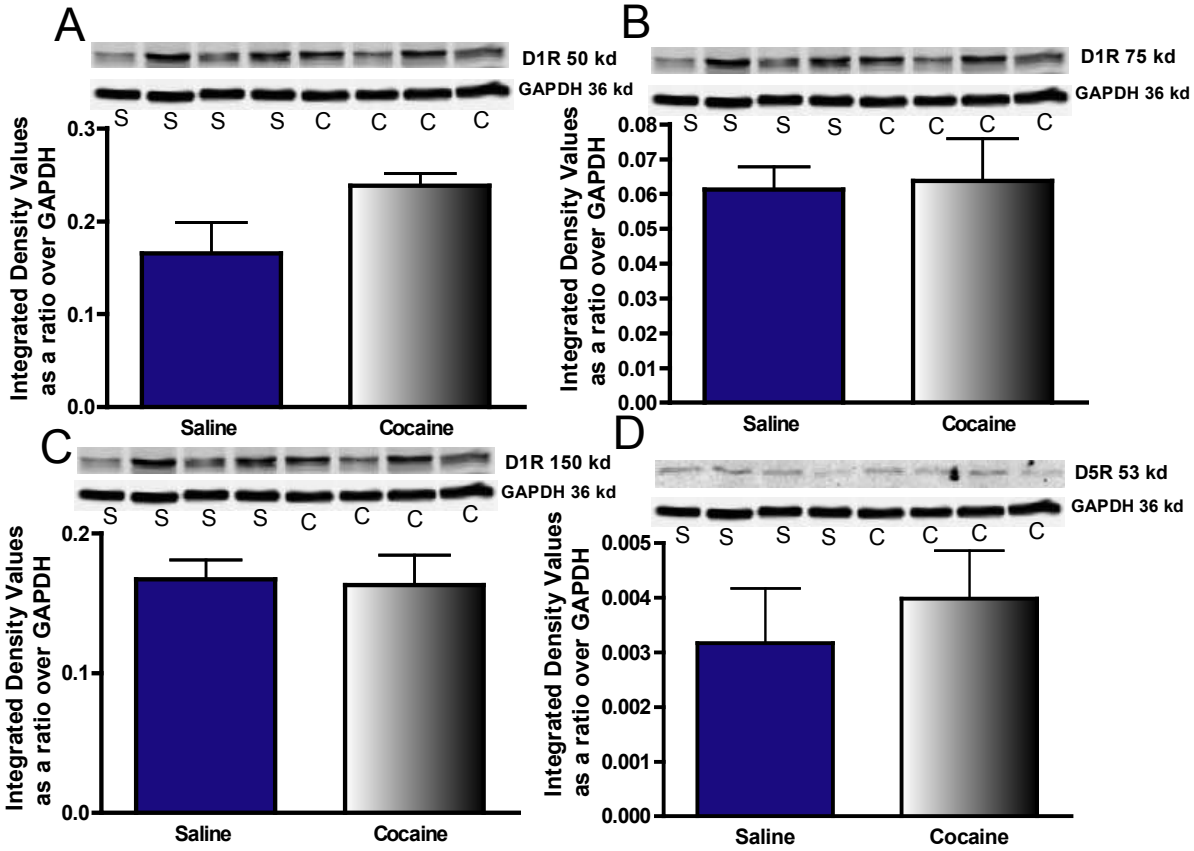

Figure 15: D1R and D5R expression in the amygdala was not significantly different in cocaine- and saline-treated animals. Graph shows protein expression changes cocaine- and saline-treated CPP animals. The upper band in the blots is D1R or D5R and the lower band is GAPDH. Panel A shows the 50 kd band for D1R. Panel B shows the 75 kd band of D1R. Panel C shows the 150 kd band of D1R. Panel D shows the 53 kd band of D5R.

Changes in dopamine receptor expression were not detected after withdrawal from cocaine.

Since electrophysiology studies showed that an SKF81297, an agonist of the D1-like receptor, induced LTP in the BLA-CeA pathway in slices from cocaine-treated animals, we analyzed D1 receptor protein expression in whole lysate of amygdala dissected from cocaine- and saline-treated animals. We were able to visualize not only the expected bands at 50 and 53 kd for D1R and D5R, respectively, but we also detected prominent bands at 75 and 150 kd for D1R, meaning that we have more visible bands

than expected. One explanation for these bands is that they are a result of different stages of glycosylation for the receptor (Bergson et al., 1995; Karpa et al., 1999). There is also a possibility that the receptors could be forming dimers which may have altered the molecular weight of the protein. We detected a slight but insignificant elevation in both D1R and D5R protein expression in the amygdala obtained from cocaine and saline-treated animals **(Figure 15A-D)**. There was no change in the expression level of the 50 kd band of D1R (t=1.795, n=4, p=0.18; **Figure 15A**). The 75 and 150 kd bands were also not significantly different between cocaine and saline groups (75: t=0.538, n=4, p=0.64; 150: t=0.126, n=4, p=0.91) **(Figure 15B and C)**. Similarly, the densities of the 53 and 75 kd bands for D5R were also not significantly altered (53: t=0.47, n=4, p=0.67) **(Figure 15D)** (data for the 75 kd band not shown). The data suggested that a change in receptor expression for either receptor could not account for the D1-induced LTP recorded in the cocaine withdrawn group.

Another possibility is that whole amygdala lysate protein samples may not be concentrated enough to detect changes in protein levels. For this reason we probed for D1R and D5R protein using membrane fractions. Western blot analyses performed on proteins obtained from membrane fractions also showed no significant difference in D1 receptor expression between cocaine and saline groups for the 50, 75 and 100 kd bands (25: t=1.01, n=5, p=0.417; 50: t=1.06, n=5, p=0.40; 75: t=0.55, n=5, p=0.64; 100: t=0.24, n=5, p=0.84) **(Figure 16A-D)**. The fact that there was no significant change in D1R expression between cocaine and saline groups in both the whole amygdala lysate and the membrane fractions suggests that the D1-mediated LTP in the BLA-CeA pathway may be mediated thorough processes that do not involve changes in D1R protein expression.

Westerns performed on protein from membrane fractions also showed no significant difference in D5 receptor expression between cocaine and saline groups for the 50 and 100 kd bands (50: t=0.14, n=5, p=0.90; 100: t=0.61, n=5, p=0.60) **(Figure 17A and B)**. Like the data for D1R, these data also suggested that changes in D5R protein expression may not contribute to the dopamine-induced LTP recorded in the BLA-CeA pathway, but does correlate with electrophysiology data showing that a D5

antagonist does not affect the dopamine-induced LTP and suggesting that D5 may not be involved in this dopamine-induced LTP.

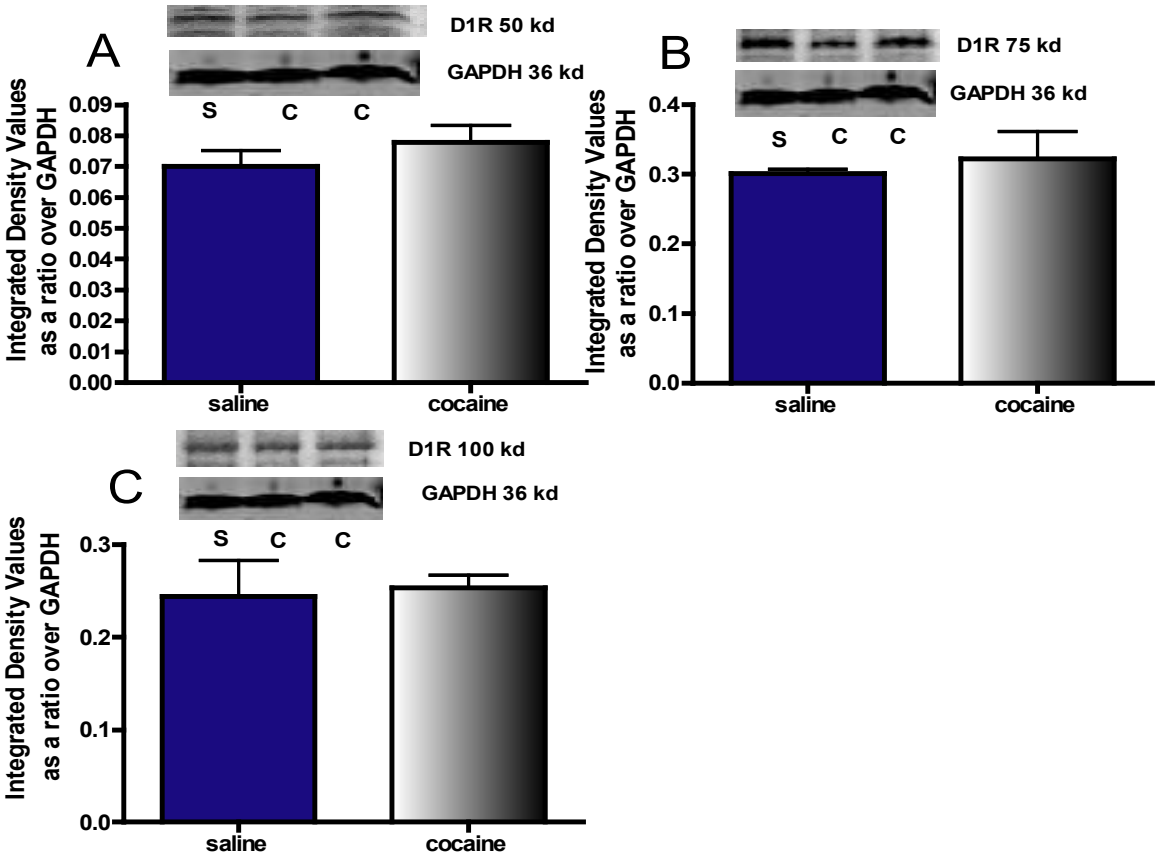

Figure 16: D1 receptor expression in the amygdala was not significantly different in the protein membrane fraction of amygdala from cocaine- and saline-treated animals. Graph shows protein expression changes cocaine- and saline-treated CPP animals. The upper band in the blots is D1R and the lower band is GAPDH. Panel A shows the 25 kd band of D1R. Panel B shows the 50 kd band of D1R. Panel C shows the 75 kd band of D1R. Panel D shows the 100 kd band of D1R.

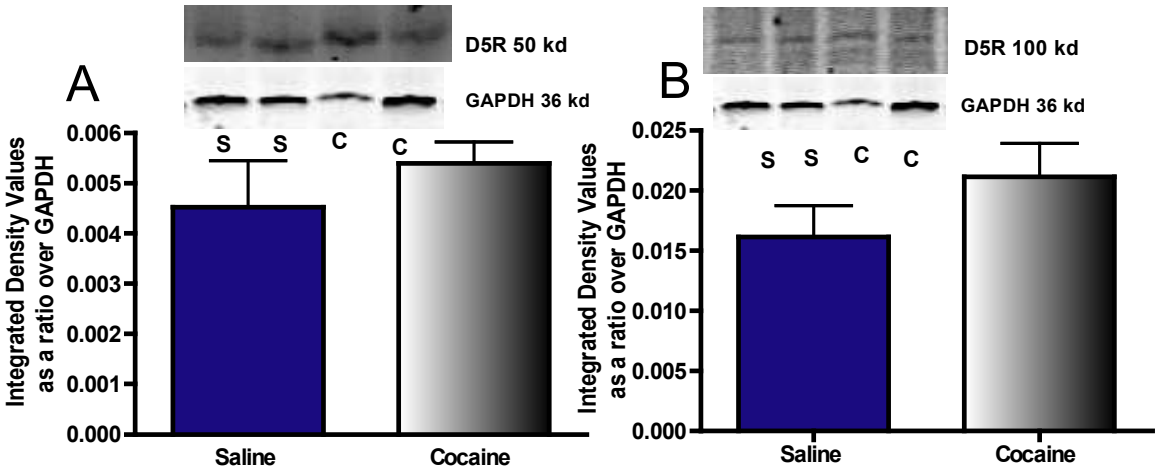

Figure 17: D5 receptor expression is not significantly different in membrane fractions of amygdala from cocaine- and saline-treated animals. Graph shows protein expression changes cocaine- and saline-treated CPP animals. The upper band in the blots is D5R and the lower band is GAPDH. Panel A shows the 50 kd band of D5R. Panel B shows the 100 kd band of D5R.

MGluR protein expression was not altered after cocaine treatment.

Electrophysiology studies showed that an antagonist selective for mGluR1, LY367385, blocked the SKF81297-induced LTP. For this reason, we analyzed the effects of cocaine treatment on mGluR1 protein expression in whole cell lysates of amygdala from cocaine- and saline-treated animals.

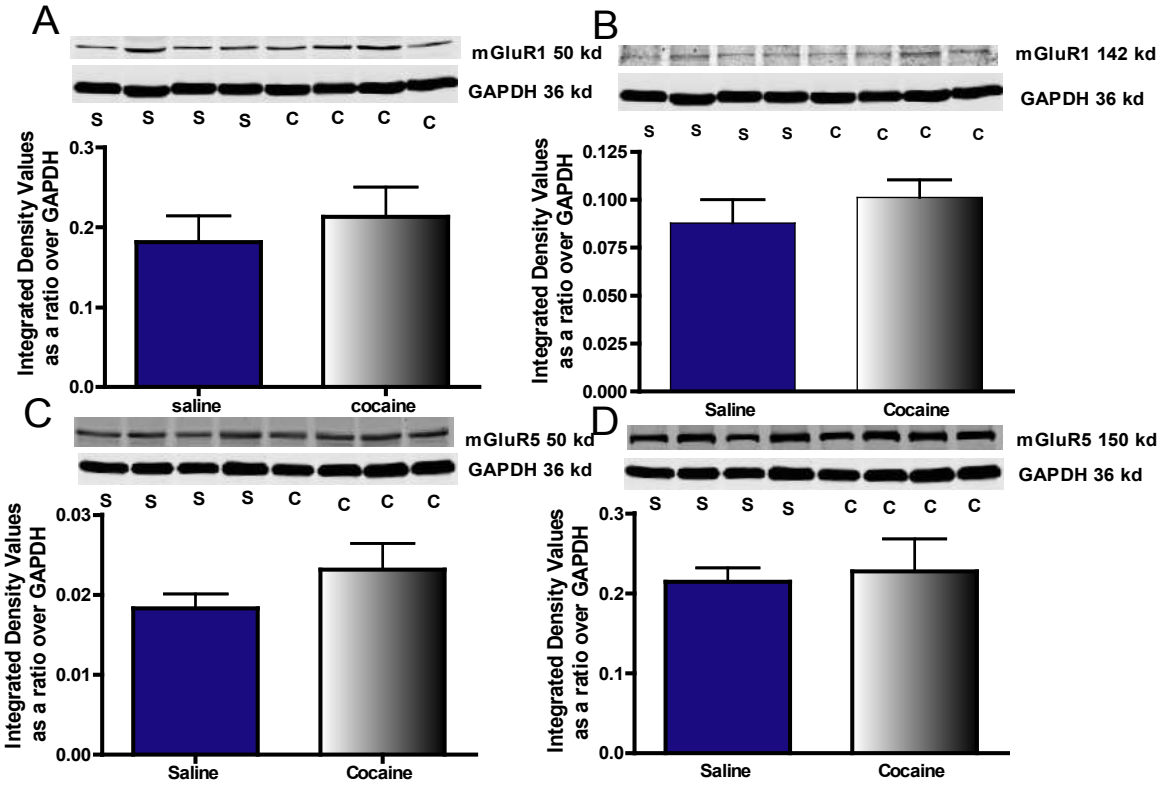

Figure 18: mGluR1 (142 kd band) and mGluR5 (150 kd band) expression in whole cell homogenates of amygdala was not changed in cocaine CPP animals. Graph shows protein expression changes in cocaine- and saline-treated CPP animals. The upper band the blots is mGluR1 or mGluR5 and the lower band is GAPDH. Panel A shows the 50 kd band of mGluR1. Panel B shows the 142 kd band of mGluR1. Panel C shows the 50 kd band of mGluR5. Panel D shows the 150 kd band of mGluR5.

The expected bands for mGluR1 are at 142 and 50 kd, and the expected bands for mGluR5 are 150 and 50 kd. The mGluR1 protein expression was not significantly increased for the 50 ($t=0.64$, $n=4$, $p=0.54$) and 142 ($t=0.87$, $n=4$, $p=0.42$) kd bands in the amygdala from cocaine and saline-treated animals **(Fig 18A and B)**. There was also no difference in mGluR5 protein expression between cocaine and saline groups in the 50 ($t=1.004$, $n=4$, $p=0.39$) and 150 ($t=0.39$, $n=5$, $p=0.72$) kd bands **(Figure 18C and D)**. The lack of a change in these receptor levels suggested that the enhanced D1-induced LTP in cocaine may not involve changes in overall receptor levels.

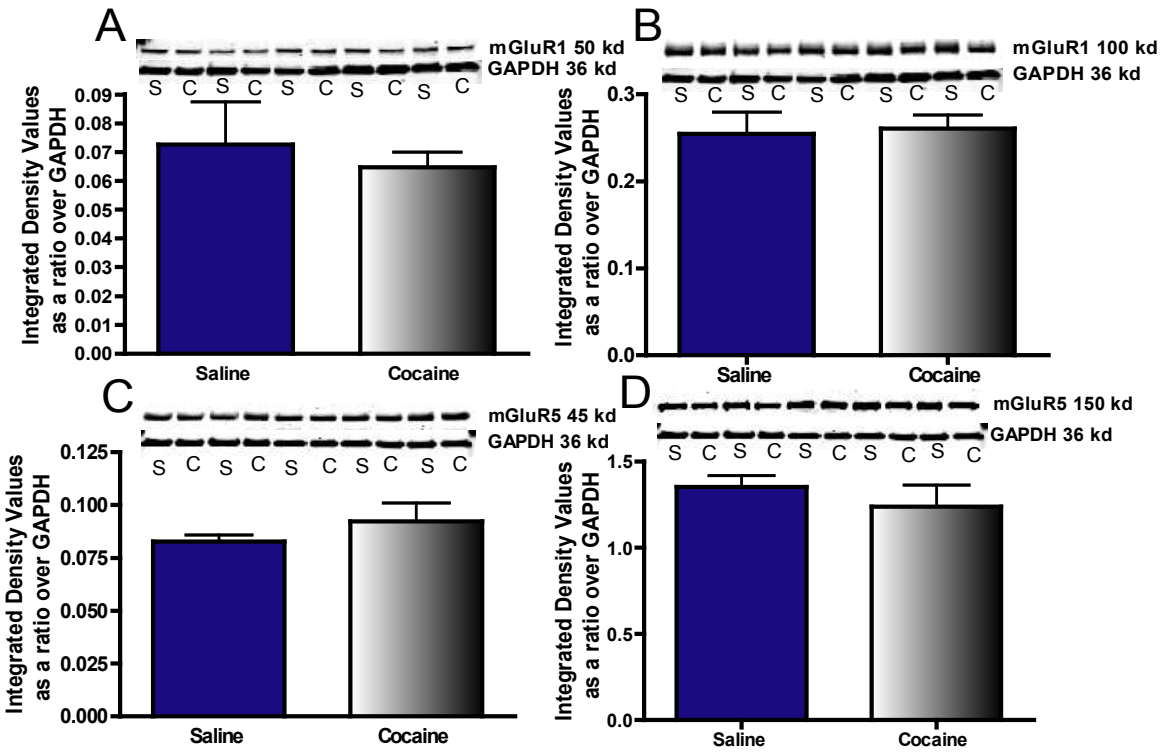

Figure 19: In the membrane fraction, mGluR1 and mGluR5 protein expression was also not significantly increased in the amygdala from cocaine-treated animals. Graph shows protein expression changes in cocaine- and saline-treated CPP animals. The upper band the blots is either mGluR1 or mGluR5 and the lower band is GAPDH. The protein is from the membrane fraction.

To analyze receptor levels further we tested membrane fractions of amygdala from cocaine- and saline-treated animals. There was no significant change in protein expression for the 50 ($t=0.43$, $n=5$, $p=0.69$) or 100 ($t=0.16$, $n=5$, $p=0.88$) kd bands of mGlur1 and the 45 ($t=1.06$, $n=5$, $p=0.16$) and 150 ($t=0.82$, $n=5$, $p=0.46$) kd bands of mGluR5 protein from cocaine and saline-treated animals **(Figure 19A-D)**. These data suggested that these receptors, which may play a role in D1-induced LTP, likely do not, involve changes in overall receptor levels.

PLD1 and PLD2 were co-immunoprecipitated with mGluR1 and mGluR5 antibodies in the cocaine CPP group.

Since the SKF-induced LTP was blocked with an antagonist of mGluR-linked to PLD, we examined whether PLD could be coimmunoprecipitated with mGluR1 or mGluR5 antibodies. PLD1 protein was co-immunoprecipitated with the mGluR1 and GluR5 antibodies in cocaine samples. The upper band around 100 kd in the cocaine lanes, number 3 and 4, was PLD1 protein. These bands were not detected in the saline lanes, number 1 and 2 **(Figure 20A)**. These data suggested that PLD1 may be linked to mGluR1 and mGluR5 in cocaine animals but not saline. When PLD2 was probed, the expected PLD2 band around 100 kd was also detected in the cocaine lanes, number 3 and 4, but not in the

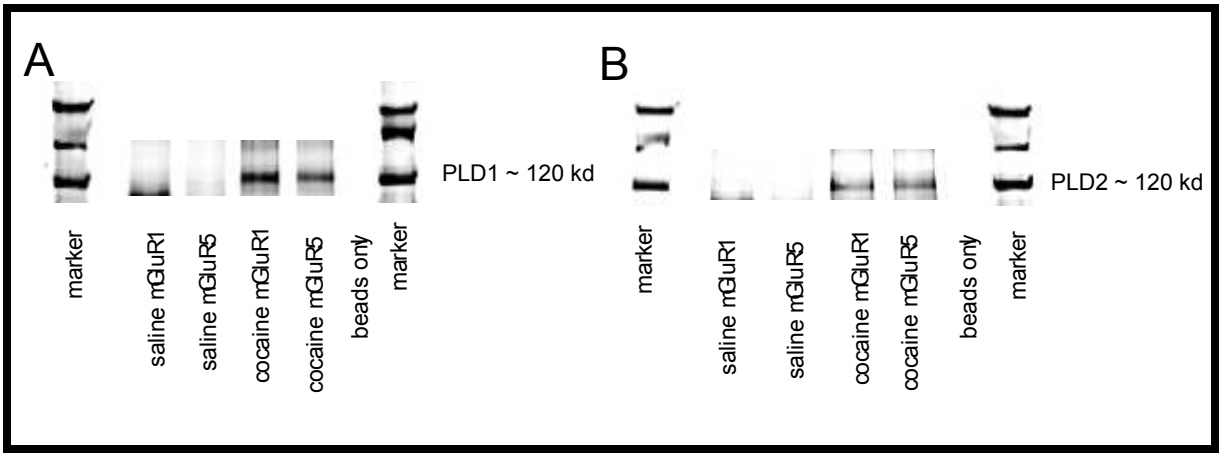

Figure 20: Amygdala PLD1 and PLD2 were coimmunoprecipitated with mGluR1 and mGluR5 antibodies in protein from cocaine -treated animals. Immunoblots show PLD1 and PLD2 were precipitated with mGluR1 and mGluR5 antibodies. In these blots 5 µl of mGluR1 and mGluR5 antibody was added to 150 µg samples of cocaine and saline protein.

first two saline lanes, indicating that PLD2 could also be co-immunoprecipitated by mGluR1 and mGluR5 antibodies, suggesting that that these two proteins may be linked in protein from cocaine- but not saline-treated animals **(Figure 20B)**. For both figures, the lower and more prominent band at 50 kd is the heavy chain IgG band and the upper band around 75 kd detected in the saline lanes is also part of the IgG band.

PLD1 but not PLD2 were immunoprecipitated with D1R and D5R antibodies.

Since the electrophysiology data showed that an antagonist of the mGluR-linked to PLD blocked the D1-induced LTP, we tested whether PLD1 and PLD2 could be co-immunoprecipitated with D1 and D5.

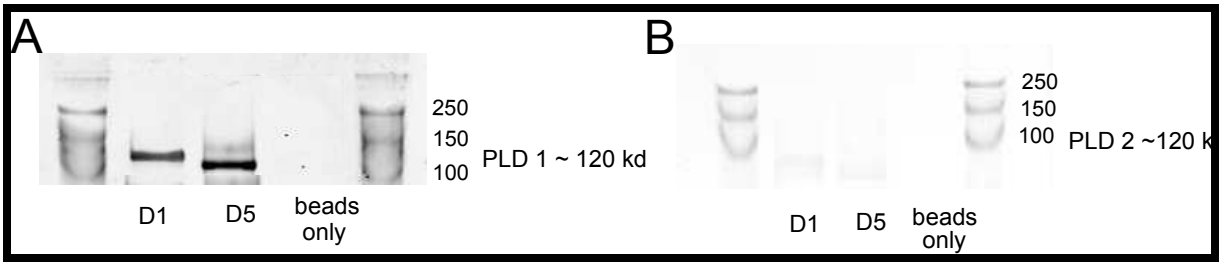

Figure 21: Amygdala PLD1 but not PLD2 was co-immunoprecipitated with D1 and D5 antibodies. Figure shows immunoblots in which PLD1 (Panel A) is precipitated and PLD2 (Panel B) is not precipitated with D1 and D5 antibodies. In these blots 5 µl of D1 and D5 antibody was added to 150 µg samples of naïve protein.

When co-immunoprecipitated with either D1R or D5R antibodies, PLD1 protein was labeled in an upper band of around 100 kD **(Figure 21A)**. These data suggested that PLD1 is linked to both D1R and D5R. The expected PLD2 band around 100 kD was not detected on the right-hand blot in **Figure 21B** suggesting that D1R and D5R proteins are not linked to PLD2 and that these two proteins may not be directly linked.

PCCG-13 and LY367385 inhibited and L-CSA stimulated PLD activity in cocaine but not saline slices.

We utilized an enzymatic activity assay (Albani-Torregrossa et al., 1999) to determine whether the increase in PLD protein in cocaine treated group reflected an increase in PLD activity. Furthermore, our electrophysiological data indicated that the D_1 receptor-induced LTP was mediated through an mGluR1 receptor. To investigate this interaction further, we next analyzed the effect of PCCG-13 and L-cysteine sulfinic acid (L-CSA) treatment on PLD activity. L-CSA is a specific agonist of the mGluR-linked to PLD (Boss, et al. 1994). Additionally, our studies have suggested that the mGluR specifically linked to PLD maybe a group I mGluR. We also tested whether the LY compound had a similar effect as PCCG-13 on PLD activity. In the PLD assays the level of PLD activity was measured by the amount of ^{3}H-labeled phosphatidylethanol formed

in slices from cocaine and saline-treated animals. We found that the PLD activity was increased during withdrawal in the cocaine CPP group compared to the saline group and CSA, the PLD selective mGluR agonist, enhanced PLD activity while mGluR antagonists reduced PLD activity.

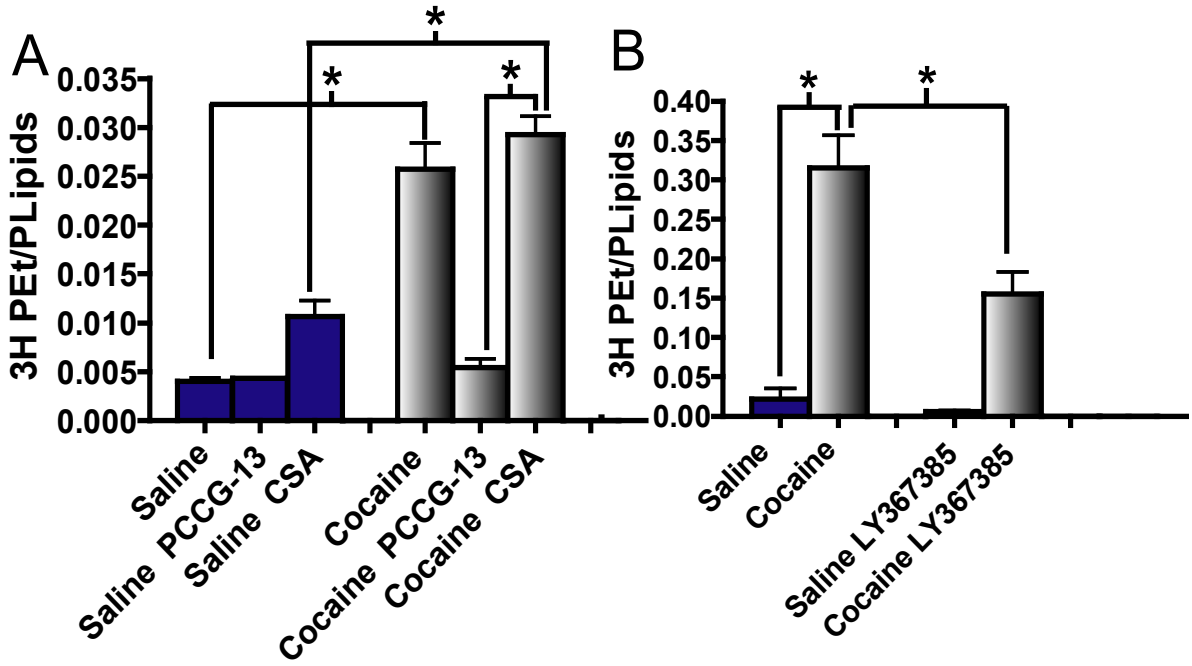

Figure 22: Cocaine treatment increased PLD activity and CSA, the agonist of the mGluR-linked to PLD, significantly stimulated PLD activity whereas the antagonist of the mGluR-linked to PLD and an mGluR1 antagonist reduced baseline PLD activity. Graph depicts the amount of phosphatidylethanol (PtdEtOH) formed under different treatment conditions as a ratio of tritiated PtdEtOH over the total amount of lipids. For both saline and cocaine groups, one sample was exposed to 2 μM PCCG-13 and another sample received 1 mM L-CSA (Panel A). Panel B shows the effects of LY367385 on PLD activity in slices from cocaine- and saline-treated animals compared to the ethanol only control group.

We found that the specific mGluR-linked to PLD played a role in the D1-mediated LTP in cocaine which was expressed as a significant increase in PLD activity in the cocaine group compared to the saline group ($t=9.451$, $p=0.0$, $n=4$). Additionally, there was a significant decrease in PLD activity in the presence of PCCG-13 compared to the cocaine control ($t=10.14$, $p=0.01$ $n=4$) **(Figure 22A)**. Furthermore, there was a

significant increase in PLD activity in the presence of CSA in the cocaine group compared to the saline CSA group (t=6.92, p=0.0203, n=4), but CSA did not significantly increase PLD activity in the cocaine group compared to the cocaine control group, which may be due to a ceiling effect on PLD activity in the cocaine control group. Our results also indicated that mGluR1 receptors play a role in the D1-mediated LTP in the cocaine group, based on the decrease in SKF81297-induced LTP in the presence of LY36738, an mGluR1 antagonist **(Figure 22B)**. We measured a significant increase in baseline PLD activity in the cocaine control group compared to saline (t=5.98, p=.0003, n=10). A decrease in PLD activity with LY367385 was also detected in amygdala slices from cocaine treated animals compared to the cocaine control (t-3.36, p=.008, n=10).

MGluR5 antagonist, MPEP, also reduced PLD Activity.

Electrophysiological data showed that the SKF-induced LTP was entirely blocked, or reduced to saline levels, in the presence of the PCCG-13, the antagonist of the mGluR-linked to PLD. The selective mGluR1 antagonist, LY367385, also blocked the SKF-induced LTP. These antagonists, PCCG-13 and LY367385, also significantly reduced PLD activity. Additionally, the SKF-induced LTP was significantly reduced in the presence of the selective mGluR5 antagonist, MPEP, compared to the SKF control. These data suggested that PLD may be linked to mGluR5 and contribute to the SKF-induced LTP. For this reason, we tested the effects of MPEP on PLD activity.

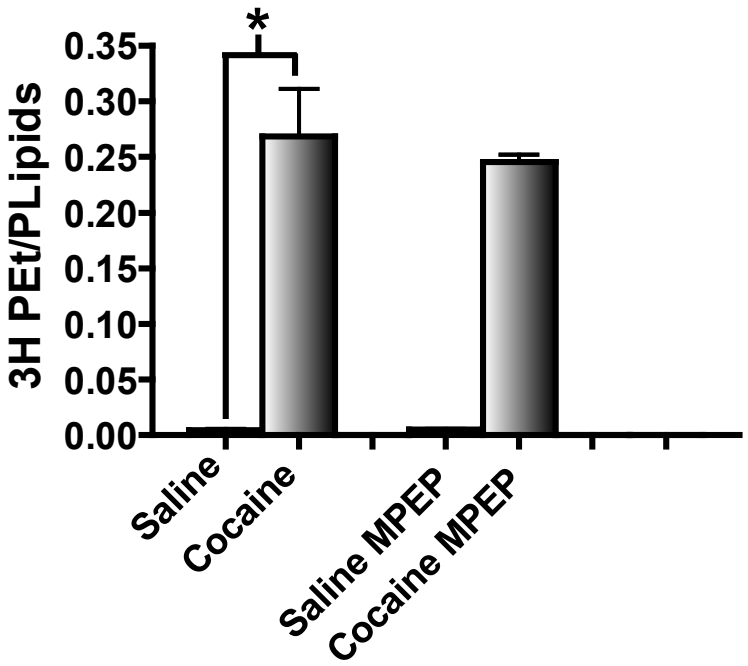

Figure 23: Cocaine treatment increased PLD activity, but an mGluR5 antagonist had no effect on PLD activity in cocaine and saline groups. Graph depicts the amount of phosphatidylethanol (PtdEtOH) formed under different conditions as a ratio of tritiated PtdEtOH over the total amount of lipids. For both the saline and cocaine goups, one sample for both exposed to 2 μM MPEP and the control sample received only ethanol.

Again, a significant increase in PLD activity measured in slices from cocaine-treated animals was measured ($t=6.84$, $n=5$, $p=0.002$) **(Figure 23)**. However, in amygdala slices from cocaine treated animals MPEP ($t=5.900$, $n=5$, $p=0.004$), there was no significant difference between the cocaine control and the cocaine group treated with MPEP. There was also no significant difference in PLD activity in the saline ethanol control and the saline group treated with MPEP ($t=0.253$, $n=5$, $p=0.81$). These data suggested that the SKF-induced LTP in cocaine animals which was blocked by MPEP was not mediated through PLD.

SKF81297 stimulated, and SCH23390 blocked PLD activity.

To examine the relationship between D1-like receptors and PLD further we next analyzed the effect of the D1 antagonist, LY367385, on PLD activity.

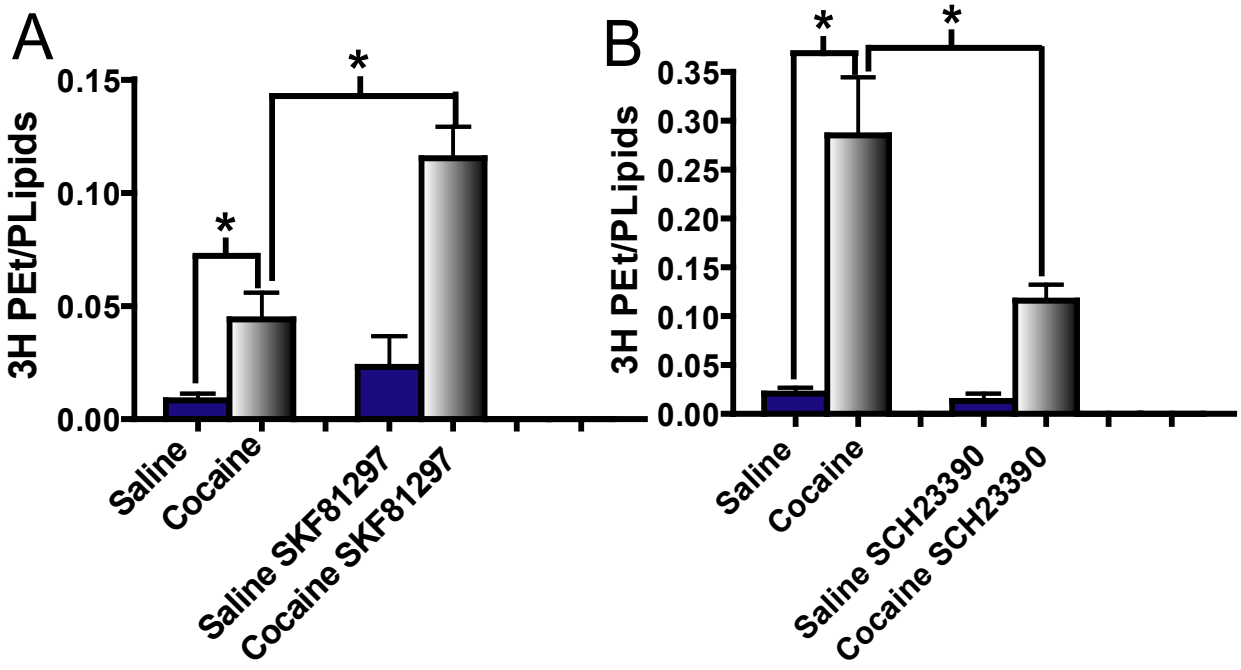

Figure 24: Amygdala PLD activity was increased by SKF81297 and decreased by SCH23390 in cocaine treated animals and PLD activity in both saline and cocaine slices. Graph depicts the amount of phosphatidylethanol (PdEthoH) formed under different treatment conditions. Panel A shows the effects of SKF81297 on PLD activity in slices from cocaine- and saline-treated animals. Panel B shows the effects of SCH23390 on PLD activity in slices from cocaine- and saline-treated animals.

Consistent with our previous data, baseline PLD activity in the cocaine treated group was significantly increased when compared to the saline control group (t=3.54, n=11; p=0.005). SKF81297 also stimulated, significantly, PLD in the cocaine (t=5.52, n=11; p=0.005) but not saline group (t=1.09, n=12, p=0.297) **(Figure 24A)**. The significant difference in the SKF81297 effect on PLD activity suggests that the increased PLD activity may play a role in the enhanced D1-mediated LTP in cocaine.

We next analyzed the effect of the D1 antagonist, SCH23390, treatment on baseline PLD activity. Again, PLD activity in slices from cocaine-treated animals compared to the saline control group (t=3.868, p=.0062, n=8) were significantly increased **(Figure 24B)**. PLD activity in slices treated with SCH23390 from cocaine-

treated animals was significantly decreased compared to the cocaine ethanol control (t=2.56, p=.037, n=8).

Effects of PCCG-13 on CPP behavior

Based on our electrophysiology results showing that application of PCCG-13 blocks the D1 agonist-induced LTP, we investigated the effects of PCCG-13 on cocaine-induced CPP behavior. We injected animals with PCCG-13 before CPP testing to determine if the molecular effects of blocking the mGluR-linked to PLD would influence CPP expression.

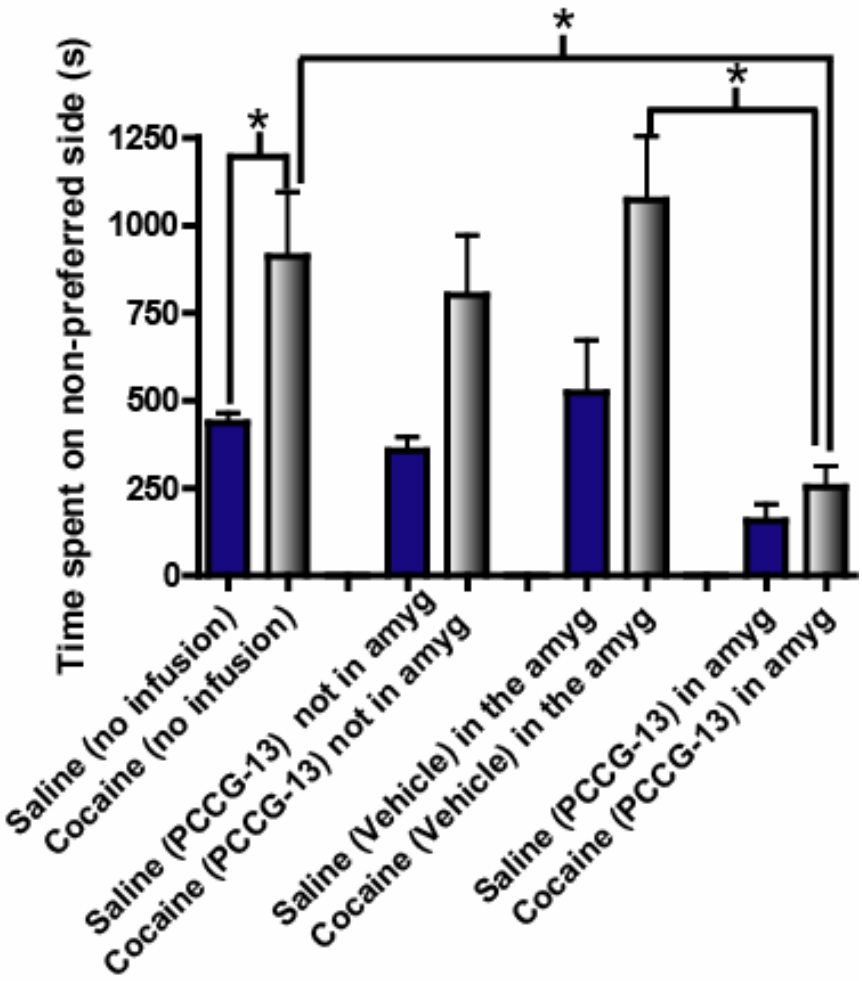

Figure 25: CPP for cocaine-treated animals blocked by injection of PCCG-13 Graph depicts time spent on preferred side for each group tested on Day 19. Animals were

injected with 2uM PCCG-13 directly into the amygdala via cannulae and then underwent CPP testing.

Animals trained in the CPP paradigm and given no injection showed that the cocaine-treated animals spent significantly more time on the non-preferred side paired with the drug and the saline-treated animals preferred the black side (t=2.45, n=4, p=0.035) **(Figure 25)**. In the second set of experiments (next pair of columns), PCCG-13 was injected into region of the brain other than the amygdala before CPP testing and while the cocaine-treated animals appeared to prefer the non-preferred side more than the saline-treated animals as in the first experiments these results were not significant (t=2.733, n=3, p=0.56). This could be due to the low number of animals tested. Additional studies should be performed to further clarify these results. In the third pair of columns, a vehicle was injected into the amygdala and again, there was no statistical significance between the preferences, even though the cocaine-treated animals appeared to prefer the non-preferred side more than saline-treated animals (t=1.69, n=3, p=0.95). These results may also require a greater number of experiments to clarify the findings. Lastly, when PCCG-13 was injected into the amygdala, both the cocaine- and saline-treated animals avoided the non-preferred side and there was no difference in preference between these groups (t=0.933, n=4, p=0.202). This reduction of preference seen in the cocaine group was also significantly reduced compared to the vehicle injection into the amygdala and the cocaine group receiving no infusion (t=3.48, n=3, p=0.04), but was not significantly reduced compared to the PCCG-13 injection not in the amygdala (t=5.26, n=4, p=0.006). However, PCCG-13 injections in the amygdala also significantly reduced their preference compared to PCCG-13 injected into other areas than the amygdala (t=7.31, n=3, p=0.08) and the saline groups receiving no injections (t=7.133, n=4, p=0.002). These results suggest that PCCG-13 may have an effect on expression of CPP in the cocaine group and may also have a small effect within the saline group, but further studies are necessary to answer additional questions.

DISCUSSION

The main findings from our studies are: (1) a D1 agonist-induced LTP in the BLA-CeA pathway was recorded during withdrawal from cocaine CPP; (2) the D1 agonist-induced LTP was mediated through a specific mGluR linked to PLD and mGluR1; (3) the D1 agonist – induced LTP was partially mediated by mGluR5 and PLC; (4) the D1-agonist induced LTP was mimicked by dopamine in the presence of raclopride and was dependent on GABAergic inhibition suggesting localization of DA receptors on GABAergic interneurons in the BLA-CeA pathway; (5) PLD activity was increased in the amygdala of the cocaine group and dependent upon DA receptor activation; (6) the increased PLD activity with cocaine CPP was also dependent on mGluR1 and the specific mGluR-linked to PLD, but not mGluR5; (7) in the amygdala of naïve animals PLD1, but not PLD2, was co-immunoprecipitated with mGluR1, D1R, and D5R 'and finally (8) cocaine CPP was blocked by the antagonist of the mGluR-linked to PLD.

D1 agonist-induced LTP in the BLA-CeA pathway was enhanced after withdrawal from cocaine.

D1 agonist-induced LTP in the BLA-CeA pathway was enhanced after two weeks withdrawal from cocaine when compared to saline control. This data agrees with previous studies in our lab showing that High-Frequency Stimulation (HFS)-LTP in saline and cocaine groups was partially mediated through D1, but not D2 receptors in the BLA-CeA pathway (Liu et al, submitted). Additionally, D1, but not D2 receptors work with glutamate receptors to induce LTP in the nucleus accumbens (Schotanus and Cherugi, 2008). D1-like DA receptors are critical for sustained enhancement of NMDA receptor-dependent LTP in the hippocampal-prefrontal cortex pathway (mPFC) (Gurden et al., 2000). This pathway involves the adenylyl cyclase and PKA signaling cascade (Gurden et al., 2000). Within the hippocampus, D1-like-mediated synaptic plasticity involves increased expression of c-Fos protein possibly controlling memory storage processing (Kang et al., 2000). Furthermore, after 4mg/kg injections of methamphetamine (MA) daily for four days, D1-like, but not D2-like receptors mediated

the impairment of hippocampal-mPFC LTP due to MA (Ishikawa et al., 2005). The Kang and Ishikawa studies showed that under normal conditions, D1-like receptors were involved in LTP for memory storage and under drug-induced conditions, the same receptors were responsible for deterioration of synaptic plasticity within the same hippocampal-mPFC pathway (Ishikawa et al., 2005). Blockade of D1-like receptors in mPFC pyramidal neurons significantly reduced the cocaine-induced enhancement of LTP (Huang et al., 2007). This occurred in tandem with a reduction in $GABA_A$ receptor-mediated inhibition in pyramidal neurons (Huang et al., 2007). Dopamine receptors, specifically D1-like, also play a role in LTP by increasing AMPA receptor surface expression on the processes of medium spiny neurons in the nucleus accumbens after psychostimulant administration (Chao et al., 2002). Within the nucleus accumbens core, co-infusion of D1-like and NMDA receptor antagonists significantly impaired instrumental learning, but the antagonists had no effect when used independently, showing that in this brain region dopamine receptors work in concert with glutamatergic receptors to meditate LTP (Smith-Roe and Kelley 2000).

Dopamine itself in the presence of a selective D5 antagonist mimicked the D1-induced LTP in amygdala slices from cocaine treated animals since the magnitudes of the responses were identical. However, the time course of the dopamine-induced LTP was more rapid than that induced by the D1 agonist. The delayed SKF-induced LTP may be due to the structure of the agonist compared to the endogenous neurotransmitter. SKF81297 is a benzazepine, which has a phenyl substituent that is functionally mobile and could affect its efficacy to bind and elicit its actions (Charifson et al., 1989; Mottola et al., 1996). In one study, comparing the effects of 13 different types of D1 partial and full agonists on cAMP production and D1R internalization, there were differences in results even between groups of agonists which had similar structures (Ryman-Rasmussen et al., 2005). Specifically, there was a difference in the amount of cAMP produced by SKF81297 compared to dopamine. Additionally, the efficacy and rate at which SKF81297 caused internalization of the D1R was similar to dopamine's effects, but was quite different than what two other benzazepine agonists produced. These data suggest

that even though a drug is an agonist for a specific receptor and produces the same results as the endogenous neurotransmitter, the rate may be different due to its binding characteristics of the different agonists to a different D1R-G-protein interface. Furthermore, the dopamine-induced LTP in the presence of raclopride was blocked completely by a D1 antagonist indicating a D1 receptor mediated LTP. Co-localization studies show that there are high levels of D1R mRNA while D2R levels are fairly low (Maltais et al., 2000) in the intercalated nuclei of amygdala which correlate with our data indicating that the dopamine-induced LTP was mediated via D1 receptors.

The D1 agonist-induced LTP was dependent on GABAergic inhibition suggesting localization of DA receptors on GABAergic interneurons in the BLA-CeA pathway.

Our studies showed that the D1 agonist-induced LTP was dependent on the level of GABAergic inhibition specifically that in the high PTX concentration (50 μM) the SKF81297-induced LTP in cocaine slices was reduced to saline control levels and when GABA inhibition was fully present the SKF-induced LTP in cocaine slices was not significantly different than in 10μM PTX. These results indicated that the mechanism responsible for the SKF-induced LTP in slices from cocaine-treated animals required GABAergic inhibition suggesting localization of DA receptors on GABAergic interneurons in the BLA-CeA pathway.

Studies show that GABAergic intercalated neurons gate feed-forward inhibition from the BLA to the CeA (Royer et al., 1999). Some investigators proposed that an increase in released dopamine acting on D1 receptors can enhance amygdala-related behaviors via a reduction in inhibition from the BLA to the CeA by enhancing firing of projection neurons (Rosenkranz and Grace, 1999). However, other studies showed that BLA interneurons are depolarized directly by D1 activation, thereby increasing inhibition (Kroner et al., 2004; Rosenkranz and Grace, 2002). Pape (2005) proposed that the balance of excitatory and inhibitory projections throughout the BLA increased excitation in the BLA-CeA pathway due to D1 activation causing dopaminergic hyperpolarization of medial intercalated cells located between the BLA and CeA pathway (Marowsky et al.,

2005) **(Illustration 3)**. Based on these studies it is likely that the block of D1-induced LTP with PTX was due to loss of intercalated cell function, leading to a reduction in feed-forward inhibition.

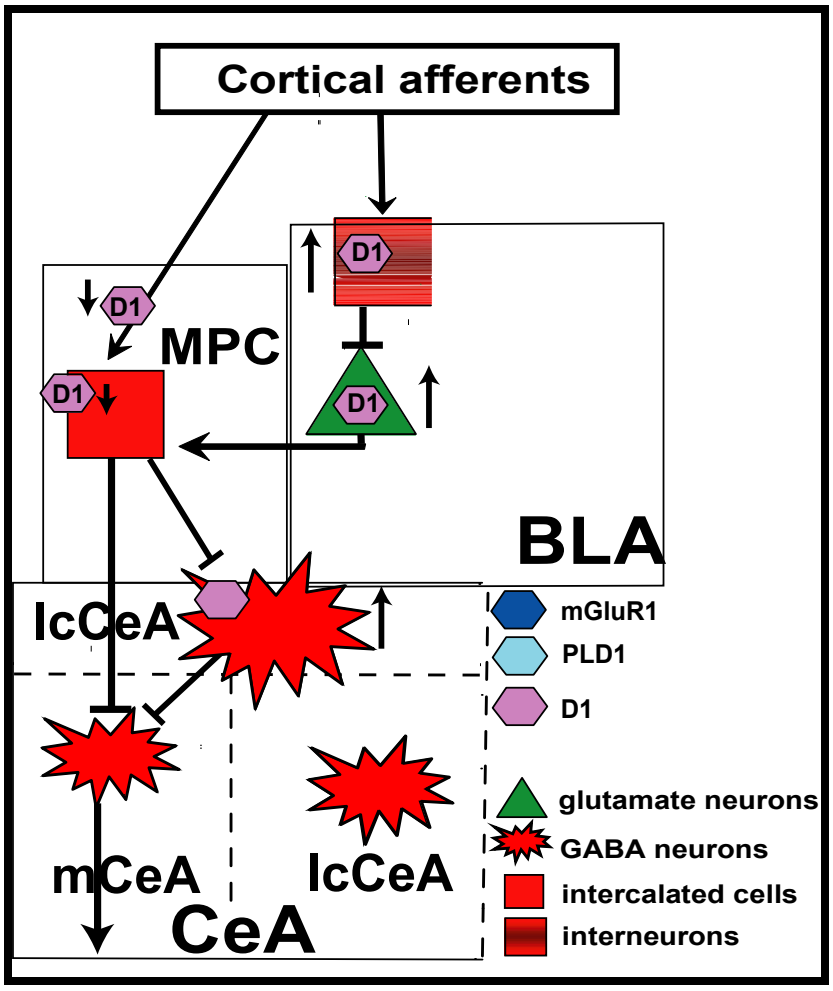

Illustration 3: Excitation in the BLA-CeA pathway. Shows that excitation of DRs on BLA projection neurons results in excitation in the CeA via decreased inhibition from GABA interneurons.

D1 agonist-induced LTP was mediated through mGluR1, mGluR5, the specific mGluR1 linked to PLD and PLC.

The D1-induced LTP in the BLA-CeA pathway was blocked by a selective mGluR1 and partially blocked by a selective mGluR5 antagonist. Using

autoradiographic labeling of the mGluR1 antagonist, the mGluR1 was localized in the amygdaloid complex (Lavreysen et al., 2004). Additionally, immunocytochemical and mRNA localization studies show that mGluR1 and mGluR5 are present at high levels in the central nucleus of the amygdala (Martin et al., 1992; Shigemoto et al., 1992; Shigemoto et al., 1993). Importantly, high levels of mGluR1alpha were found in the small intercalated cell masses in the capsular division of the central nucleus of the amygdala (Agassadian and Cassell, 2008). Furthermore, moderate levels of mGluR5 receptors have been detected in the BLA and low to moderate levels were measured in both the medial and lateral paracapsular intercalated cells within the amygdala (de la Mora et al., 2006). Due to the fact that both dopaminergic and glutamatergic receptors are located within the amygdaloid complex, numerous investigators have searched for a possible link between these two receptor types in mediating addiction and other anxiety related disorders. D1, but not D2 receptors, have been shown to work cooperatively with both mGluR1 and mGluR5 receptors to induce LTP in the nucleus accumbens (Schotanus and Cherugi, 2008). Additionally, in behavioral studies DHPG, a group I mGluR antagonist, blocked locomotor behavior induced by agonists of D1-like, but not D2-like receptors in the nucleus accumbens (Rouillon et al., 2008). Although other brain regions have been investigated for these links, we were the first to report the interaction between dopamine and group I mGluRs in the amygdala after withdrawal from cocaine.

Importantly, PCCG-13, the selective antagonist for the mGluR linked to PLD, blocked the D1 agonist-induced LTP. Studies have shown a link between DA (both D1-like and D2-like) receptors and PLD in HEK 293 cells (Senogles, 2000, Everett and Senogles, 2004), in cardiac tissue (Yasunari et al., 2000), and in Aplysia neurons (Kawasaki et al., 2008). However, a link between these receptors has not been found in the mammalian brain. We showed that PCCG-13, the antagonist for the mGluR linked to PLD, blocked the SKF81297-induced LTP in the BLA-CeA pathway, suggesting a functional link between the two receptors in mediating the long-term changes within the amygdala that are the present during withdrawal from cocaine.

Lastly, our data revealed that the D1 agonist-induced LTP in the BLA-CeA pathway was also partially mediated by the second messenger, PLC. It is well-documented that D1-like receptors are positively linked to adenylate cyclase (Monsma et al., 1990), but other studies have shown that D1-like receptors are also linked to PLC and inositol triphosphate (IP3) formation (Undie et al., 2000). Interestingly earlier work showed that the DA receptors were not linked to adenylate cyclase, but rather coupled to some other second messenger system within the amygdala (Undie and Friedman, 1990).

Increased PLD activity in the cocaine group was dependent upon DA receptor activation.

Because we showed that, electrophysiologically, PLD was a critical part of the D1 agonist-induced LTP, we further investigated the D1 receptor actions mediating this PLD response to withdrawal in the cocaine CPP group. PLD is activated by glutamate in neurons (Rujano, et al. 2004) but not in astrocytes and it can also be activated by the excitatory amino acid, L-cysteine sulfinic acid (L-CSA), which is structurally similar to L-glutamate and L-aspartate and is thought to be an endogenous agonist for the mGluR linked to PLD (Boss, et al. 1994). In addition to being activated by glutamate, PLD activity can also be affected by dopamine. D2-like antagonists blocked PLD activation (Senogles, 2000), while, in HEK 293 cells, D2-like agonists specifically stimulate PLD2 (Senogles, 2003). Additionally, the D3 agonist (+)7-OH DPAT increased PLD activity in HEK 293 cells, which was blocked by D3 antagonists (Everett and Senogles, 2004).

We demonstrated that PLD activity was increased in the presence of the selective D1 agonist, SKF81297, in the cocaine group compared to cocaine control. Furthermore, PLD activity was decreased in the cocaine group compared to the cocaine control after application of the selective D1 antagonist, SCH23390. These findings suggested that baseline PLD activity in the cocaine group maybe dependent on constituitive D1 activation. Previous studies showed that D5 receptor stimulation with fenoldapam, decreased PLD2, but not PLD1 activity in HEK-nD_5R cells (Yang et al., 2005). Furthermore, activation of $D1_A$ (D1R) and $D1_B$ (also known as D5R) receptors prevents

platelet-derived growth factor containing 2 β peptide chains (PDGF-BB)-induced oxidative stress in vascular smooth muscle cells via PLD suppression (Yasunari et al., 2000). Another group showed that PLD, downstream of RhoB, may regulate D1-like receptor-induced Na^+ current in *Aplysia* neurons by controlling endocytosis and exocytosis of receptors (Kawasaki et al., 2008).

Our results suggested that the increased dopamine release due to withdrawal from chronic cocaine (Trang-Nguyen et al., 1998) may increase the enzymatic activity of PLD. This interaction could occur because of a linkage between D1R and PLD or by actions downstream of the dopamine receptors.

PLD activity was increased in cocaine- treated animals and dependent on mGluR1, and the specific mGluR-linked to PLD, but not mGluR5

Our research showed that PLD activity was enhanced after cocaine withdrawal. While there is no previous data showing a relationship between PLD and cocaine, there are studies showing that PLD2 may interact with and regulate trafficking of the μ-opioid receptor in response to morphine (Koch et al., 2003). Additional studies show that PLD2 interacts with epidermal growth factor (Slaaby et al., 1998), and plays a role in regulating clathrin-coated vesicle-mediated endocytosis of G-protein coupled receptors (Koch et al., 2003; Bhattacharya et al., 2004; Du et al., 2004). The fact that PLD2 interacts with the membrane receptor EGF to mediate a function supports our theory that PLD is linked to mGluR1 and interacts with D1 receptors to mediate the D1 agonist-induced LTP.

Currently, it still remains unclear whether PLD is linked to a known type of mGluR or a unique type reserved only for PLD function. Boss and Conn (1992), as well as other studies (Holler et al., 1993; Boss et al., 1994; Sarri et al., 1995; Pellegrini-Giampietro et al., 1996; Klein et al., 1997), showed that agonists and antagonists for ionotropic glutamate receptors did not have an effect on PLD, suggesting that PLD is indeed linked to an mGluR. Therefore, we tested mGluR agonists and antagonists found to be specific based on previous data regarding PLD and mGluR interactions. Although the characteristics of the type of mGluR linked to PLD has not been rigorously classified,

there is an antagonist for the specific mGluR-linked to PLD, PCCG-13, (Albani-Torregrossa, 1999). We found that PCCG-13 significantly decreased PLD activity in the cocaine group.

The nonselective mGluR agonist, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate [(1S,3R)- ACPD], was able to stimulate PLD activity in the hippocampus of adult rats (Boss and Conn, 1992) as well as in neocortical and striatal tissues (Schoepp and Conn, 1993). The selective group 1 mGluR agonist, (RS)-3,5-dihydroxypheylglycine (DHPG) (Schoepp, et al. 1994), was able to stimulate PLC activity in hippocampal slices, but inhibited ACPD-induced PLD activity in the hippocampus (Pelligrini and Giampietro et al., 1996). However, a different group showed that in the hippocampus of, young, 8-day-old rats, DHPG activated PLD and the group I mGluR antagonist, methyl-4-carboxyphenylglycine (MCPG), inhibited the PLD response to glutamate indicating a linkage of PLD to mGluR1 or mGluR5 (Klein, et al. 1997). The same group showed that group II and III mGluRs are probably not linked to PLD because the group II mGluR agonist, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine (DCG-IV), very weakly stimulated PLD activity and antagonists of group III mGluRs were ineffective on PLD activity. Because more recent data suggested that PLD was linked to a group I mGluR, we tested the effects of LY367385, a selective mGluR1 antagonist, on PLD activity and found that it significantly reduced PLD activity in slices treated with LY367385 compared to the cocaine control. This result indicated that the mGluR linked to PLD is an mGluR1 subtype, but we also tested the effects of MPEP, a selective mGluR5 antagonist, and found that there was no difference between slices treated with MPEP and the cocaine control. These data suggest that PLD is linked to an mGluR1-like subtype.

Over time, theories regarding PLD have evolved from thinking that it is linked to a unique type of mGluR into the idea that it is linked to a group I mGluR and this specific receptor interaction my be dependent on both developmental stage and tissue type. This is suggested by studies performed in hippocampal, neocortex, and striatal tissues as well as cerebrocortical synaptosomes but many of the studies report results that conflict with others. Studies performed in cerebrocortical synaptosomes have shown that both ACPD

and DHPG stimulated PLD activity and the addition of phorbol esters enhanced the ACPD effect (Shinomura et al., 2000). Furthermore, the PKC inhibitor, GF109203X, did not inhibit PLD activity, and there was no significant increase in phosphoinositide-specific phospholipase C (PI-PLC) or diacylglycerol (DAG) by ACPD after only 15 seconds, indicating that PLD activity occurs in a PKC- and PI-PLC- independent manner (Shinomura et al., 2000). While their studies indicate that PLD is linked to group I type of mGluR, some studies in hippocampal slices showed that ACPD, and a group I mGluR agonist, AIDA (1-aminoindan-1,5-dicarboxylic acid) but not DHPG, could stimulate PLD activity (Pellegrini-Giampietro et al., 1999). These same studies also showed that the group I mGluR antagonist, MCPG, was actually able to stimulate PLD activity, thereby conflicting with other data suggesting a linkage between PLD and group I mGluRs. In another study performed in hippocampal slices MCPG did not have an effect on PLD activity, but ACPD could simulate PLD activity (Boss et al., 1994), suggesting a lesser role for mGluR5 compared to mGluR1 because MCPG has been shown to act equally on mGluR5 than mGluR1 (rabbet et al., 1995), while ACPD activity favors mGluR1 over mGluR5 (Moroni et al., 1997). These data show that while there may be differing results between tissue type, the over-riding evidence indicates that PLD is not linked to a new type of mGluR, but rather a group I mGluR1.

Our studies are unique because we focused on PLD activity in the amygdala after cocaine addiction and none of the previous studies have evaluated PLD activity after cocaine withdrawal or in the amygdala. In the studies listed above, PLD activity was induced using ACPD, L-CSA, or glutamate and effects of different antagonists were evaluated on the agonist-induced PLD activity. Our studies are unique in that for our PLD activity assays we tested the effects of various drugs on basal PLD activity after cocaine CPP which could elicit different results than drug-induced responses. Also, we are the first to report the effects of the selective mGluR5 antagonist, MPEP, on PLD activity. An important remaining question is whether these PLD actions occur pre- or post-synaptically.

PLD1, but not PLD2, mGluR1, mGluR5, D1R, or D5R expression was increased in amygdala protein from cocaine-treated animals.

Although D1 receptor-mediated activity was altered on withdrawal from cocaine, no significant difference in protein expression was measured in amygdale from animals treated with cocaine. Similar findings were reported when an increase in phosphoinositide-3-kinase (PI3-K) activity was measured in striatal neurons after acute amphetamine treatment, but the protein level was unchanged (McGinty et al., 2008). Our data suggest that the increase in dopamine release (Trang-Nguyen et al., 1998) and the dopamine-induced LTP recorded in the amygdala after cocaine was not due to an increase in overall D1 receptor expression but rather to an increase in PLD1 activity and expression. The fact that both PLD expression and activity were increased after cocaine suggests that PLD may interact with D1 to mediate the D1 agonist-induced LTP.

Since group 1 mGluRs were shown to mediate the D1-induced LTP, we also performed westerns to determine whether or not these actions were the result of changes in mGluR1 protein expression after withdrawal from cocaine. Our results showed that there was no significant change in either mGluR1 or mGluR5 protein expression. However, the protein concentration may have been too dilute to be detected in whole amygdale lysate, so we subsequently probed membrane fractions. Although, mGluR1 and mGluR5 protein expression was unchanged after cocaine, the mechanism by which these two receptors mediate the D1 agonist-induced LTP could be the result of the increase in PLD activity. One such mechanism could include PLD physically connecting D1R and the mGluRs. This point is discussed further in the following sections.

PLD1 and PLD2 expression increased, but mGluR1, mGluR5, D1R, and D5R were unchanged in fractionation protein from cocaine- compared saline-treated animals

Because we did not identify a significant change in receptor expression for whole amygdala lysate, we also analyzed fractionated amygdala protein to determine if the changes in receptor expression could only be detected at the level of the plasma membrane. While we did see a significant increase in both PLD1 and PLD2 expression

in membrane protein, we still did not measure a significant difference in receptor expression for D1R, D5R, mGluR1, or mGluR5 in the membrane fraction of amygdala protein.

We measured a physical link between PLD1 and mGluR1/5 and D1/5 receptors. The membrane changes that occur due to cocaine could involve heterodimerization instead of an increase in overall receptor expression. Some investigators found that non-functional receptors require dimerization to be functional, and functional ones can dimerize to increase function and both can be triggered by agonists (Jordan and Devi, 1999; Marshall et al. 1999), or in our case, cocaine, glutamate, or dopamine release. It is known that mGluR5 dimerizes via its disulfide bonds in its N-terminal extracellular (Romano et al. 1996). It is possible to detect heterodimerization in westerns unless heterodimerizing mGluRs and DRs interfere with antibody binding. Formation of mGluR and DR heterodimers could explain the increased LTP recorded in cocaine CPP amygdala with only an increase in PLD1 and PLD2 expression and no change in receptor expression.

PLD1, but not PLD2, was co-immunoprecipitated with D1R and D5R in naïve protein.

PLD1 and PLD2 are highly ubiquitous enzymes that are involved in brain development, anti-apoptotic pathways, neurodegeneration prevention, neurite formation, endo- and exocytosis, and formation of choline for acetylcholine formation (for reviews: Jones et al., 1999; Cockcroft, 2001; Klein, 2005). PLD interacts with numerous types of proteins and pathways to accomplish its functions, but no one has shown that PLD can be co-immunoprecipitated with mGluRs or dopamine receptors in brain tissue. Trang-Nguyen and colleagues (Trang-Nguyen et al, 1999) found that PLD2 but not PLD1 could be pulled-down with mGluR1 antibodies, but these studies were performed in an expression system, not in brain tissue (Bhattacharya et al., 2004).

We found that PLD1, but not PLD2, could be positively detected on a blot containing naïve amygdalar protein incubated with D1R and D5R antibodies. This

finding indicated that the two D1-like receptors may be physically linked to PLD1, but not PLD2, is a novel finding **(Illustration 4)**. D5 receptors were shown to be involved in a protein-protein interaction between γ_2 subunits with $GABA_A$ receptors in HEK cells which resulted in a mutually inhibitory functional interaction between the two receptors (Liu et al., 2000) suggesting that DA receptors can be physically linked to other receptors to mediate a particular signaling function. Although the D1R and not D5R played a role in the DA-induced LTP in the BLA-CeA pathway, the fact that the D5R has been shown to be physically linked to GABA receptors suggests that other DA receptors could also be involved in similar events and could explain why the dopamine-induced LTP was dependent upon PLD function and PLD activity is increased after dopamine stimulation. Additionally, because the D5- γ_2-GABA receptor interaction described above was inhibitory, we can propose that dopamine receptors involved on intercalated interneurons in the BLA-CeA pathway inhibit GABAergic transmission (Marowsky et al., 2005) and that the interaction with $\gamma_2$subunits may account for induction of LTP in those cells.

PLD1 and PLD2 were both co-immunoprecipitated with mGluR1 and mGluR5 in protein from cocaine- but not saline-treated animals

We are the first group to show that both PLD1 and PLD2 could be positively probed on a blot containing amygdalar protein from cocaine-treated animals incubated with mGluR1 and mGlur5 antibodies. These results indicated that PLD1 and PLD2 could be physically linked to mGluR1 and/or mGluR5 receptors **(Illustration 4)**. Interestingly, there was no pull-down in protein detected in saline-treated animals, only in cocaine suggesting a cocaine-induced alteration in PLD-mGluR interaction.

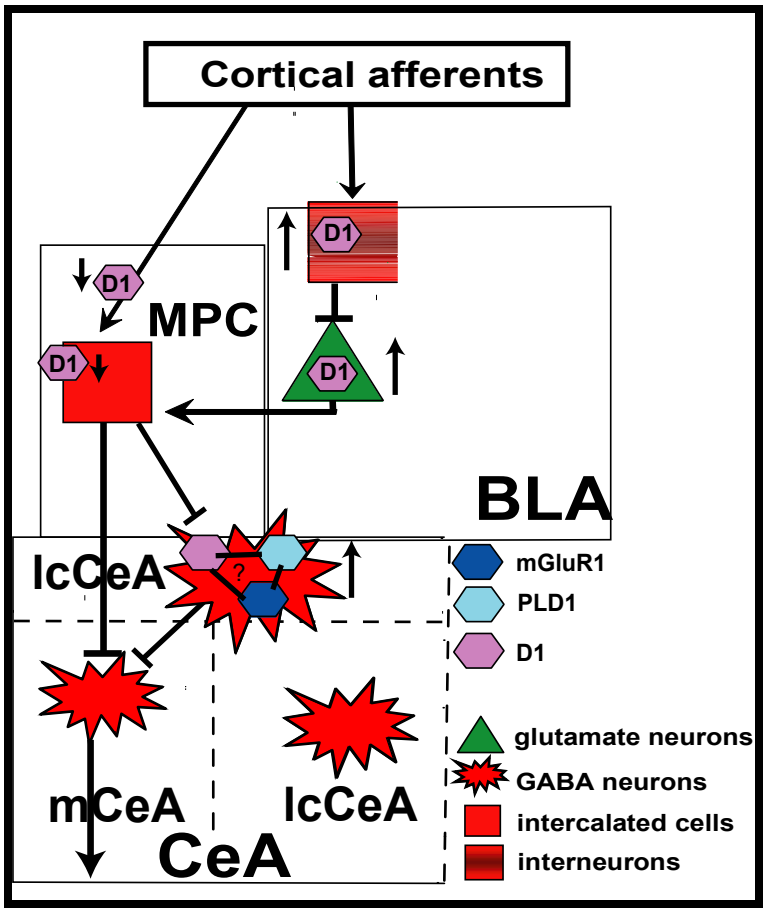

Illustration 4: BLA-CeA pathway signaling. Diagram depicts the final proposed schematic for the location and interaction of the receptors mediating the SKF81297-induced LTP.

Cocaine CPP was blocked by the antagonist of the mGluR-linked to PLD, PCCG-13

We have shown that when given the choice between two sides of a chamber, rats prefer the side on which injections of cocaine were given even though this side is not the naturally preferred side for the animals. Animals receiving saline injections prefer the dark side of the chamber regardless of which side this injection is given. Because PCCG-13 was able to block the D1 agonist-induced LTP, we tested the effects of PCCG-13 on a functional behavioral scale to determine the outcome of injecting rats with PCCG-13 through a cannula in their amygdala on CPP. We found that injecting rats with PCCG-13 before undergoing CPP testing significantly blocked the preference of cocaine injected

animals for the non-preferred side compared to the cocaine injected animals tested without PCCG-13 injections. Preference for the non-preferred side in animals receiving saline injections was also significantly reduced compared to testing saline injected animals in the absence of PCCG-13 injections. This suggests that blocking the mGluR-linked to PLD reduced the cue-induced craving for cocaine administered before CPP testing, indicating that the mGluR-linked to PLD plays a major role in the pathway mediating craving after chronic cocaine administration. The fact that the preference of saline injected animals was also significantly reduced suggests that the mGluR-linked to PLD may also play a role in maintaining basal levels of activity in the amygdala.

Conclusion

These studies provide evidence for the mechanisms associated with cocaine CPP and withdrawal. . Our results suggest an interplay of multiple receptors in downstream signaling pathways resulting in LTP and possibly CPP. The electrophysiological studies suggest that a D1 agonist-induced LTP is not only dependent upon GABA inhibition, but also mediated by the mGluR-linked to PLD, mGluR1, and partially by mGluR5 and PLC. A possible explanation for these results is that increased dopamine and glutamate during cocaine withdrawal results in activation of both dopamine receptors and group I mGluRs. Since we showed that PLD1 could be pulled-down by mGluR1 and mGluR5 antibodies in cocaine slices but not saline ones, and PLD1 could also be pulled-down by D1 and D5 antibodies, PLD1 could by the common factor that links these two pathways after cocaine treatment. Interestingly, some investigators suggest that increasing PLD activity without affecting PI-PLC generates a more stable signaling pathway whose time course is more suitable for long term potentiation (Klein et al., 1995), which may also provide an explanation of why D1 agonist-induced LTP can be recorded in cocaine- but not saline-treated animals

Further Studies

Our studies have indicted a pivotal role of the mGluR-linked to PLD in the D1 agonist-induced LTP. The next step would be to show that PLD, itself, is the critical

protein in this mechanism. Since there are no antibodies available to directly antagonize or activate PLD, we would need to utilize a viral vector system to directly manipulate the level of PLD in mammalian neurons. This knock-down or knock-out system could be used to test the effects of low or no levels of PLD on the D1 agonist-induced LTP in the BLA-CeA with electrophysiology recordings. Furthermore, rats could be injected with siRNA for PLD directly into the amygdala before CPP testing to directly test the effects of amygdala PLD on CPP behavior.

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VITA

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