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THE ROLE OF AMYGDALA GROUP I MGLURS IN SYNAPTIC PLASTICITY AND CONDITIONED PLACE PREFERENCE IN RODENTS

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THE ROLE OF AMYGDALA GROUP I MGLURS IN SYNPATIC PLASTICITY AND CONDITIONED PLACE PREFERENCE IN RODENTS

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For my father, Craig, who has always taught me that education is the only thing that can never be taken away from you

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THE ROLE OF AMYGALA GROUP I MGLURS AND DOWNSTREAM SIGNALING MOLECULES IN THE EXPRESSION OF CONDITIONED PLACE PREFERENCE IN RODENTS

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Currently, there are no approved medications for treating cocaine addiction, and cocaine addicts are highly prone to relapse. Cocaine cravings or seeking is difficult to control because of contextual cues and adequate treatment is not available. Previous studies found that group I metabotropic glutamate receptor (mGluR) antagonists can block the induction of conditioned place preference (CPP), a measure of cocaine seeking behavior, but their functions after clinically relevant withdrawal periods are not known. Our study showed that group I mGluR antagonists failed to block the expression of cocaine-induced CPP. Furthermore, the amygdala is known to be involved in the learned associations between cocaine and the cocaine-taking environment; changes in these associations are reflected in an in vitro model of plasticity, long-term potentiation, in amygdala pathways. Chronic cocaine withdrawal did not affect mGluR5-mediated LTP in the basolateral to central amygdala pathway. However, mGluR1-mediated LTP was reduced after cocaine administration and withdrawal and was partially due to GABA inhibition via endocannabinoids.

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INTRODUCTION

Currently, there are no approved medications for treating cocaine addiction, and cocaine addicts are highly prone to relapse (Kleber, 2005; Washton and Stone-Washton, 1990). Relapse vulnerability is related to long-term adaptations in the brain that bias the individual's behavior towards addiction (Nestler, 2001). Research has revealed three types of stimuli that can trigger drug-seeking and –use after abstinence: contextual cues, pharmacological stimuli, and exposure to stressors (Kalivas and McFarland, 2003). Therefore, determining neural mechanisms underlying the effects of these triggering factors is critical for the development of effective treatments that reduce relapse vulnerability in cocaine addicted subjects. Our research focuses on cocaine associated contextual cues in the amygdala. As part of the brain's limbic system, the amygdala is involved in the formation of stimulus-reward associations and in the processing of conditioned cue associational information (Aggleton, 2000).

Glutamate transmission plays a key role in synaptic plasticity and cocaine addiction (Kalivas, 2004). Specifically, literature indicates that Group I metabotropic glutamate receptors (mGluRs) are involved in behavioral responses to cocaine. Group 1 mGluRs, composed of mGluR1 and mGluR5 subtypes, couple via G_q to phospholipase C and adenylyl cyclase via G_s and, when activated, lead the activation of protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein (MAP) kinase kinase (MEK). Additionally, activation of Group I mGluRs triggers the release of endocannabinoids (eCBs), which act as retrograde synaptic modulators to inhibit neurotransmitter release (Wilson and Nicoll, 2002).

Activation of Group I mGluRs can induce long term potentiation (LTP) or long term depression (LTD), the mechanisms thought to be responsible for learning and memory. Chronic cocaine administration can disrupt LTP or LTD. Whole cell patch clamp recordings showed that chronic cocaine exposure altered the sensitivity of group II and group III mGluRs in the central amygdala (Neugebauer et al., 2000). In mice, mGluR5-mediated LTD in the bed nucleus of the stria terminalis was reduced after

multiple exposures to cocaine whether self- or investigator-administered (Grueter et al., 2006). A single cocaine exposure abolished LTD in the nucleus accumbus (NAc) and was correlated with a reduction in mGluR5 surface expression (Fourgeaud et al., 2004). However, the role of mGluR1 induced synaptic changes following chronic cocaine administration and withdrawal has not been investigated.

To address this question we explored the role of amygdala group I mGluRs and downstream signaling molecules in the expression of cocaine-induced conditioned place preference (CPP) in rats. We found that chronic cocaine withdrawal did not affect mGluR5-mediated LTP in the basolateral to central amygdala pathway. However, mGluR1-mediated LTP was reduced after cocaine administration and withdrawal compared to saline treated control animals. We tested the hypothesis that mGluR1-mediated LTP worked through interneurons thereby reducing gamma-aminobutyric acid (GABA) inhibition onto glutamatergic neurons. Our results showed that mGluR1-mediated LTP was partially due to GABA inhibition via endocannabinoids, was N-methyl-D-aspartic acid (NMDA) independent, and was not dependent on PKC, PKA, or MEK.

BACKGROUND

Conditioned place preference

Frequently, drug relapse occurs because of the ability of drug-associated cues to induce craving (Brabant et al., 2005), and craving is more intense during protracted withdrawal (Gawin, 1991). CPP is a behavioral paradigm used to study the association between drug exposure and environmental cues because it models cue-elicited conditioning that may motivate drug taking behavior (Bardo et al., 1995). A drug treatment (and its internal effect) is paired with the external stimuli of one environment (Carr et al., 1989). During a test session, the animals are given the choice between both environments, and if the drug-treated animals recognize the stimuli in that environment and spend more time there, CPP has occurred (Carr et al., 1989). We used the CPP paradigm to study the effects of pharmacotherapeutic agents that may modulate cue-induced reinstatement of cocaine-seeking behavior via the amygdala.

The amygdala and cocaine

The amygdala is part of the brain's limbic system and plays a role in stimulus-reward associations and in the association between environmental cues and conditioned information processing (Aggleton, 2000). The amygdala is composed of three major nuclei: the basolateral nucleus (BLA), the lateral nucleus (LA), and the central nucleus (CeA). Lesions to the BLA prevented drug-associated cue responses during withdrawal from cocaine in self-administering animals (Meil and See, 1996). Additionally, amygdaloid lesions caused a complete blockade of cocaine-induced CPP (Brown and Fibiger, 1993). Other studies have shown that BLA and CeA inactivation affected acquisition and expression of conditioned relapse to cocaine (Kruzich and See, 2004; See et al., 2003). Furthermore, brain imaging studies showed that the amygdala was activated in the presence of cocaine-related stimuli (Bonson et al., 2002).

MGluRs and cocaine

Glutamate receptors are divided into two main groups: ionotropic glutamate receptors and metabotropic glutamate receptors. The eight existing mGluRs are further divided into 3 groups based on sequence homology, second messenger coupling, and pharmacology (Kew and Kemp, 2005). Group I contains mGluR1 and mGluR5 subtypes. Group II is composed of mGluR2 and mGluR3 while group III includes mGluR4, and mGluRs 6-8. Group I mGluRs couple via G_q to phospholipase C and adenylyl cyclase via G_s whereas group II and III mGluRs couple via G_i/G_o to inhibit adenylyl cyclase activity.

MGluRs linked to phosphoinositide hydrolysis, such as the metabotropic group I receptors, desensitize in the prolonged presence of agonists or during repetitive stimulation (Rodriguez-Moreno et al., 1998), which is likely to protect against acute and chronic over-stimulation (Dhami and Ferguson, 2006). According to Dhami and Ferguson (2006) the desensitization process involves receptor phosphorylation, arrestin binding and internalization. Thus, alterations in group I mGluRs function may significantly affect excitatory synaptic transmission.

The role of mGluRs in drug addiction has been investigated with behavioral and electrophysiological studies. Group II mGluRs have been implicated in cocaine cue-associated return to drug seeking (Baptista, 2004). Whole cell patch clamp recordings showed that chronic cocaine exposure altered the sensitivity of group II and group III mGluRs in the central amygdala (Neugebauer et al., 2000). In mice mGluR5-mediated LTD in the bed nucleus of the stria terminalis was reduced after multiple exposures to cocaine whether self- or investigator-administered (Grueter et al., 2006).

It is possible the synthesis or density of mGluR proteins is altered after chronic cocaine and possibly after withdrawal. Swanson and co-workers (2001) found decreased protein levels for both Homer1b/c and mGluR5 in the NAc three weeks after cocaine treatment. However, 24 hours after chronic cocaine administration mGluR5 and extracellular regulated kinase (ERK) 1/2 proteins were unchanged in the bed nucleus of the stria terminalis (Grueter et al., 2006). Moreover, incubation of cocaine craving was

mediated by time-dependent increases in ERK signaling in the CeA in response to cocaine cues (Lu et al., 2005), suggesting that ERK in the CeA may influence mGluR1 or 5-mediated effects. Additionally, the group I specific mGluR agonist (RS)-3,4-dihydroxyphenylglycine's (DHPG) that normally induces glutamate release showed reduced capacity to do so after repeated cocaine administration (Swanson et al., 2001). MGluR5 mRNA levels increased in the NAc after repeated cocaine injections and 3 weeks of withdrawal (Ghasemzadeh et al., 1999) as did mGluR2/3 protein levels in the nucleus accumbens and the prefrontal cortex (Xi et al., 2002). However, another study showed that a single exposure to cocaine caused a decrease in mGluR5 surface expression in the NAc (Fourgeaud et al., 2004). Differences in mGluR5 synthesis or expression may reflect neuroadaptive changes in glutamatergic transmission after acute vs. chronic cocaine administration.

Changes in the modulatory actions of mGluRs on synaptic transmission are likely to have an effect on behavior. For example, numerous behavioral studies on cocaine and mGluRs indicated an overall reduction in self-administration in both knockout and antagonist related studies. MGluR5 knockout mice did not self-administer cocaine although food self-administration was unaffected, nor did they show increased locomotor activity (Chiamulera, 2001). Likewise, the mGluR5 antagonist (negative allosteric modulator), 2-methyl-6-(phenylethynyl)-pyridine (MPEP), dose dependently reduced cocaine self-administration in rats (Kenny et al., 2003) and monkeys (Lee et al., 2005) while having no effect on food intake. These data indicated that mGluR5s regulate motivation for drug consumption while they do not affect natural reinforcement. Furthermore, the non-selective mGluR agonist, ±-trans-1-amionocyclopentane-1,3-dicarboxylic acid (t-ACPD), caused an increase in cocaine-induced hyperactivity (Dunn et al., 2005) whereas a group III mGluR agonist blocked cocaine induced hyperactivity (Mao and Wang, 2000). Taken together, these data suggest that metabotropic glutamate receptors play a key role in the effects of chronic cocaine.

MGluR5 modulators

The first identified mGluR agonists lacked specificity and possessed low affinity. Following their discovery, novel ligand synthesis provided more potent and selective group II mGluR agonists and antagonists and identified a few group I agonists and antagonists (Kuhn et al., 2002). Using direct acting mGluR agonists for therapy has proved a challenge due to receptor desensitization and adverse effects from excessive receptor activation (Marino and Conn, 2006). Thus, in the mid 1990's, investigators turned their attention to searching for non-amino acid-like modulators.

Allosteric ligands are defined as any compound that exerts its effect on a receptor through a binding site that is topographically distinct from the binding site of the endogenous ligand. These ligands are termed allosteric *modulators* if they do not have any efficacy in the absence of the orthosteric ligand. (Ritzen et al., 2005). Such modulators may enhance or inhibit effects of orthosteric ligands and accordingly, are termed positive or negative modulators. Neutral allosteric modulators block the allosteric binding site from allosteric modulators but have no effect on orthosteric ligand induced receptor signaling. It has been shown that mGluR5 is negatively modulated by MPEP and positively modulated by 3,3'-difluorobenzaldazine (DFB), *N*-{4-chloro-2-[1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl] phenyl}-2-hydroxybenzamide (CPPHA), and 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (CDPPB) (O'Brien et al., 2003; O'Brien et al., 2004; Kinney et al., 2005).

Evidence has shown that MPEP has a role in modulating the effects of cocaine. MPEP acts noncompetitively at the sixth transmembrane domain (TM6) and the third transmembrane domain (TM3) (Malherbe et al., 2003) to prevent receptor activation thereby decreasing glutamate stimulated phosphoinositide hydrolysis (Kuhn et al., 2002). MPEP significantly attenuated cue-induced cocaine reinstatement in rats and monkeys (Backstrom and Hyytia, 2006; Lee et al., 2005) and blocked LTP in the thalamic-amygdala pathway (Lee et al., 2002). Furthermore, in rats trained to self-administer cocaine, MPEP decreased the break point, implying that mGluR5 was essential in the mediation of reinforcing and incentive motivational properties of cocaine (Paterson and

Markou, 2005). Based on these results, MPEP may represent a potential therapeutic agent in the treatment of cocaine addiction.

MGluRs and CPP

Metabotropic glutamate receptors have been implicated in the expression of CPP. Blocking group II metabotropic glutamate receptors in the NAc of mice impaired CPP (Gerdjikov and Beninger, 2006). On the contrary, mGluR2 knockout mice showed enhanced cocaine-induced CPP (Morishima et al., 2005). Blocking mGluR5 with MPEP dose-dependently reduced the development of cocaine-induced CPP in mice (McGeehan and Olive, 2003). In contrast, Herzig and Schmidt (2004) showed that in rats only a high dose (50mg/kg i.p) of MPEP fully blocked morphine-induced, but not cocaine-induced, CPP, and that the lower dose (10mg/kg i.p.) did not block either. Additionally, MPEP inhibited the acquisition and expression of morphine-induced CPP in mice (Popik and Wrobel, 2002; Aoki et al., 2004). Altogether the data suggest that mGluRs may have some role in CPP, but the results are contradictory.

DHPG and long term depression/potentiation

DHPG is a group I specific mGluR agonist capable of inducing both LTP and LTD in different brain regions. In the CA1 region of the hippocampus, (RS)-DHPG induced LTD that was reversed by the mGluR antagonist (s)-MCPG (Palmer et al., 1997). (RS)-DHPG also induced LTD in the dentate gyrus (Camodeca et al., 1999). Recently it has been shown that blockade of mGluR1 or mGluR5 alone had no effect on (RS)-DHPG-induced LTD induction in the CA1 region, but the expression was reduced by 50% when mGluR1was blocked (Volk et al., 2006). DHPG is also capable of inducing LTP. In the medial vestibular nuclei of the rat, DHPG induced LTP which was blocked by the mGluR1 antagonist, CPCCOEt (Grassi et al., 2002). DHPG-induced LTP was recorded in the rat prelimbic cortex (Morris et al., 1999), in the dentate gyrus (O'Leary and O'Connor, 1999; O'Leary and O'Connor, 1997) and in the CA1 neurons (Doherty, 2000; Zho et al., 2002). The mGluR5 antagonist LY344545 blocked induction but not expression of the (S)-DHPG-induced LTP in the CA1 neurons (Doherty et al., 2000). Zho and co-workers (2002) showed that (S)-DHPG caused LTP in the CA1 region as

well as depotentiation, which was mediated by mGluR5. These data suggested that DHPG was an effective inducer of LTP and LTD.

MATERIALS AND METHODS

Animals

Male albino Sprague-Dawley rats (Harlan, Houston, TX) aged 3-4 weeks at arrival were used for all experiments. Animals were housed 2-4 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*. Animals were acclimated to the environment for 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 apparatus

Four acrylic animal chambers (16'' x 16'' x 12'') were contained inside of sound and light attenuating environmental control boxes (Accuscan Instruments, Inc, Columbus, OH). The chamber was subdivided into two distinct compartments, one with white floors and walls and a textured floorboard (raised Plexiglas bars), the other with black floors and walls and a smooth floorboard. The light intensity in the chambers was 320 lumens. On baseline and testing days, the animal was placed in a removable animal holding chamber (6'' x 3'' x 6'') that was inserted centrally between the black and the white compartment, and then raised, to allow the animal to freely roam both sides. Activity and time spent on each side was measured using the VersaMax activity monitor system (Accuscan Instruments, Inc, Columbus, OH). On conditioning days, a centrally inserted removable 12'' acrylic single pane wall restricted the animal to one compartment. During all sessions only one animal was in each chamber.

Behavioral procedures

Each CPP experiment consisted of three phases: baseline, conditioning, and testing. For baseline measurements, 24 hours prior to the first injection, one animal per chamber was allowed to freely roam both sides of the activity box for 30 minutes to test for the animal's preference for one side over the other (i.e., biased design). Animals were

randomly assigned to be cocaine treated or saline treated control animals. Animals were conditioned for the next 5 days receiving two injections daily separated by 5 hours. In the morning, cocaine treated animals were given saline (1ml/kg of 0.9% saline solution, i.p.) and restricted to the preferred black compartment of the chamber for 30 minutes. Saline treated animals were also given saline in the morning and restricted to the preferred black compartment for 30 minutes. In the afternoon, cocaine treated animals were given cocaine (15mg/kg in 1ml/kg 0.9% saline solution, i.p.) and were restricted to the non-preferred white compartment for 30 minutes. Saline treated animals were again injected with saline and were restricted to the non-preferred white compartment in the afternoon. During all afternoon conditioning sessions two additional cues were included in the environmental control chambers: a flashing light (320 lumens, on 15 seconds, off 15 seconds) for 5 minutes and a 70 decibel white noise sound pulsing once per second for 5 minutes. On the sixth day after the first injection, all animals were tested and again allowed to roam both sides of the boxes for 30 minutes (test day 6). During the test session the light and sound cues were given. Two weeks after their last injection (test day 19) another test session was performed in the presence of the sound and light cues to detect a persistence of CPP after two weeks of chronic cocaine withdrawal. A CPP score was calculated by subtracting the time spent on the drug paired side during baseline from the time spent on the drug paired side on the test day. For CPP animals used in further experiments, brain slices were prepared two weeks after the last injection.

Slice preparation

Animals were decapitated and 500μm coronal brain slices were prepared in ice-cold (0-4º C) aerated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) of the following composition (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. No anesthetics were used before decapitation to avoid their potential influence on neuronal plasticity. Brain slices were kept at room temperature for 2 hours and then submerged in a tissue bath at 32 º C ± 1 º C and allowed to equilibrate for 30 minutes. ACSF was oxygenated with carbogen (95% O_2/5% CO_2) to maintain a pH ~ 7.4.

Electrophysiology

A concentric bipolar stimulating electrode was placed in the BLA and evoked field excitatory postsynaptic potentials (fEPSPs) were recorded in the CeA. fEPSPS were evoked at a frequency of 0.05Hz. Recordings were made using a tungsten electrode (3-4 MΩ). The stimulation intensity was adjusted during each recording to evoke 50% of the maximal fEPSP response. All recordings were made in the presence of 10μM picrotoxin unless otherwise specified, and ACSF was continuously perfused at a rate of 2 ml/min.

Drugs

Cocaine HCl was a gift from the National Institute of Drug Abuse. Cocaine (15mg/mL) was dissolved in 0.9% saline solution and 15mg/kg injected intraperitoneally (i.p.). MPEP HCl was purchased from Ascent Scientific (Weston-super-Mare, United Kingdom) and was dissolved in 0.9% saline solution. JNJ16259685, DFB, APV, PD98059, BIS-1, U73122, H-89, AM251, NBQX, LY367385, and DHPG were purchased from Tocris Bioscience (Ellisville, MO). PTX was purchased from Sigma Aldrich (St. Louis, MO). For *in vitro* experiments drugs were superfused in the ACSF at their final concentrations.

Statistical analysis

Behavior

The time spent on each of the two sides per box was determined for each individual rat during the 30 minute testing period, and data is reported as a CPP score, which is the mean time ± SEM in seconds of time spent on the drug paired side on the test day minus time spent on the drug paired side during baseline preference determination. The confirmation of the biased design data is presented as the mean time on the preferred and on the non-preferred sides. All data were analyzed using t-tests and statistical significance was defined as $p<0.05$.

Electrophysiology

Analysis was performed using Clampfit 9.0 software (Axon Instruments, Inc., Sunnyvale, CA). After filtering the individual traces, the fEPSP slope was measured. For each recording, responses were averaged for statistical analysis during the last 10 minutes after washout of the drug. Data were normalized to baseline responses and analyzed using t-tests or one-way ANOVA. Statistical significance was defined as $p<0.05$.

RESULTS

Establishment of a biased CPP apparatus

Animals were allowed to roam both sides of the testing chamber for 30 minutes (1800 sec) for their baseline recording. Time spent on each side was recorded, and data is reported as the mean time ± SEM in seconds. Rats spent significantly less time on the white side (594.6 ± 62.0s) compared to the black side (1205.0 ± 62.0s) (paired t test: $t=4.951$, $p<0.01$, $n=24$) confirming that our setup was a biased design (**Fig. 1A**). Similar results were found in all subsequent experiments (data not shown).

Conditioned place preference persisted for 2 weeks after withdrawal from chronic cocaine

Following five days of conditioning, animals were allowed to roam both sides of the chamber for a 30 minute test on day 6 and then again after two weeks of withdrawal on day 19. The time spent on each side was recorded. Data is reported as a CPP score, which is the mean time ± SEM in seconds of time spent on the drug paired side on the test day minus time spent on the drug paired side during baseline preference determination (Shimosato and Watanabe, 2003; Shimosato and Ohkuma, 2000). Therefore, a negative CPP score indicates that a population of animals tested spent less time on the drug-paired side during the testing session than during the baseline session, suggesting a place preference did not develop. In contrast, if a population of animals spent more time on the drug-paired side than during the baseline session the score will be positive, suggesting a place preference. Cocaine treated rats had a significantly higher CPP score on both day 6 (240.3 ± 103.0s, $n=12$) and on day 19 (204.4 ± 136.4s, $n=12$) compared to saline treated rats (day 6: -298.4 ± 146.1, $n=12$; unpaired t test: $t=3.014$, $p<0.05$; day 19: -286.2 ± 141.0s, $n=12$; unpaired t test: $t=2.500$, $p<0.05$) (**Fig 1B)**. Hence the cocaine treated animals actively sought out the cocaine paired environment, indicating CPP. These data also suggested that cocaine-induced CPP persisted for two weeks after withdrawal from cocaine administration.

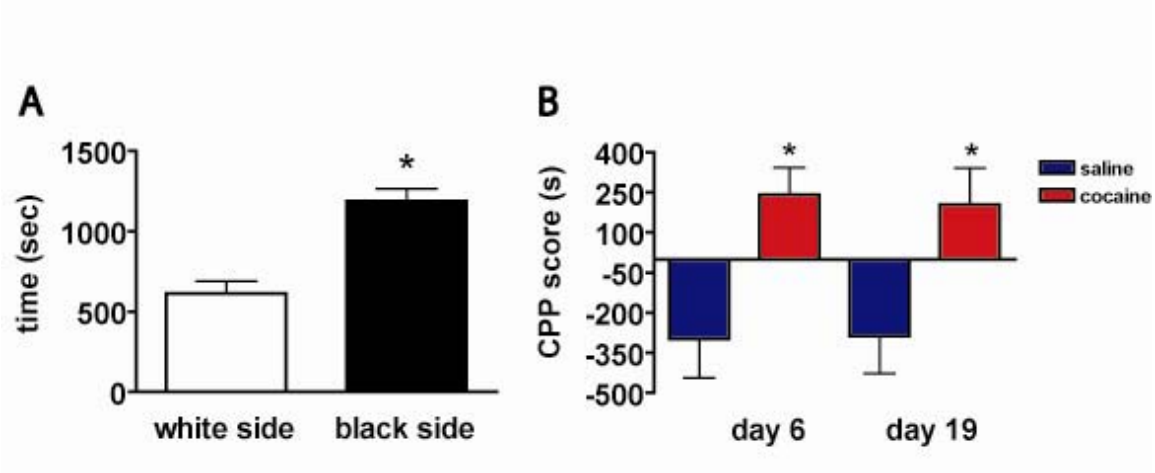

Figure 1. Establishment of a CPP paradigm. A. Under low light conditions rats preferred black rather than white-colored chamber: a biased CPP design. Bar graph depicts time spent (sec) on each side of the testing chamber (white vs. black). Rats spent significantly less time on the white compared to the black side (n=24), *p<0.01. B. CPP persisted for two weeks following termination of cocaine administration. Bar graph describes the CPP score for saline and cocaine treated rats. Cocaine treated rats had significantly greater CPP scores on both day 6 and day 19 compared to saline treated rats (n=12). *p<0.05.

Effects of MPEP on CPP

We next investigated the effects of an mGluR5 antagonist on cocaine-induced CPP. After 5 days of cocaine and saline treatments followed by a 2 week withdrawal period, animals were given MPEP (1mg/kg, i.p.) 10 minutes prior to the day 19 testing session. On test day 6 cocaine treated rats had a significantly greater CPP score (224.0 ± 68.1s) as compared to saline treated rats (-302.9 ± 96.6s; unpaired t test: t=4.460, p<0.05, n=24).

On test day 19 cocaine treated rats (165.9 ± 141.8s, n=7) had a higher CPP score than saline treated rats (-125.4 ± 135.1s) but it was not significant (unpaired t test: t=1.488, p>0.05, n=7) (**Fig 2A**). The lack of a significant difference between cocaine-induced CPP on test days 6 and 19 (in the presence of MPEP) may be due to a higher variability recorded. Because MPEP acts as a robust anxiolytic (Ballard et al., 2005), animals may not have exhibited a strong preference for one side of the chamber, leading

to higher variability. These results led us to test a higher MPEP dose on a second group of animals.

We repeated the MPEP study using a 3mg/kg dose. Ten minutes prior to the testing session animals were given 3mg/kg MPEP (i.p.) (**Fig 2B**). All test day 6 data was pooled with previous sets of animals. Cocaine treated rats had a significantly higher CPP score (224.0 ± 68.1s) compared to saline treated rats (-302.9 ± 96.6s; unpaired t test: $t=4.460$, $p<0.05$, $n=24$) on test day 6. However, as seen in our previous MPEP experiment, both saline and cocaine animals showed large variability in CPP scores from the test day 19 session. On test day 19 cocaine treated rats (329.0 ± 272.0s) showed a higher CPP score compared to saline treated rats (-155.2 ± 158.4s), but it was not significant (unpaired t test: $t=1.538$, $p>0.05$, $n=5$).

To examine if the prior effects of MPEP were due specifically to the presence of the drug we measured CPP in the absence of MPEP in the same animals 5 hours later that same day (**Fig 2C**)**.** On same day (day 19) cocaine treated rats with no MPEP showed a significantly higher CPP score (511.6 ± 244.7s) compared to the saline treated rats (-437.8 ± 78.0s) (unpaired t test: $t= 3.697$, $p<0.05$, $n=5$). The animals showed a significant CPP despite having been in the chambers 3 times without receiving cocaine, suggesting that the effects of MPEP were reversible and that extinction of the cocaine-induced CPP did not play a major role during a repeated exposure to the test environment. This also suggests a persistence of the cocaine-chamber association. Finally, these data suggested that the 3mg/kg dose of MPEP given 10 minutes before the test session reduced place preference in all animals whether it be the natural preference for the black side as shown in saline animals or the conditioned preference for the white (drug-paired) side shown in cocaine treated animals suggesting a non-specific effect. This may be due to the potent anxiolytic effect of MPEP (Brokin et al., 2002) which can, like benzodiazepines (Meririnne et al., 1999), reduce CPP.

Effects of an mGluR1 antagonist on CPP

We also examined the role of the other group I receptor subtype, mGluR1, in the expression of CPP using an mGluR1 antagonist, JNJ16259685. On test day 6, cocaine

treated rats showed a significantly higher CPP score (172.4 ± 78.5s) compared to saline treated rats (-119.0 ± 104.3s, unpaired t test: $t=2.232$, $p<0.05$, $n=32$). After a 2 week withdrawal period, animals were given JNJ16259685 (2.5mg/kg, i.p.) 10 minutes prior to the day 19 testing session. On test day 19 cocaine treated rats (-31.6 ± 421.7s) and saline treated animals (507.4 ± 236.0s) showed highly variable CPP scores that were not significantly different from each other (unpaired t test: $t=1.115$, $p>0.05$, $n=8$) (**Fig 2D**). JNJ16259685 at this dose had a nonspecific effect on the animals as shown by the large variability seen in both groups. Decreasing anxiety may cause a lack of discrimination of sides as previously seen in rats given MPEP (Perez de la Mora et al., 2006). Observations by the experimenter showed that animals simply moved to one side and lay down for the duration of the testing session. Further experiments should be conducted with both MPEP and JNJ16259685 injected into the amygdala to avoid potential non-specific effects.

DFB induced LTP in the BLA-CeA pathway in slices from both cocaine and saline treated animals

When paired pulse stimulation was applied in the presence of DFB, a mGluR5 positive allosteric modulator, LTP of field excitatory postsynaptic potentials (fEPSP) (156.9 ± 12.4% of baseline, n= 6) was induced in the BLA-CeA pathway of drug naïve rats (**Fig 3A**). Data showed that single pulse stimulation in the presence of DFB induced a smaller potentiation of the fEPSP slope compared to baseline (128.3 ± 6.8%, n=11; data not shown). Since the mechanism of DFB action is to modulate the receptor in the presence of endogenous transmitter, we used paired stimuli for all experiments involving DFB to increase the level of glutamate at the synapse.

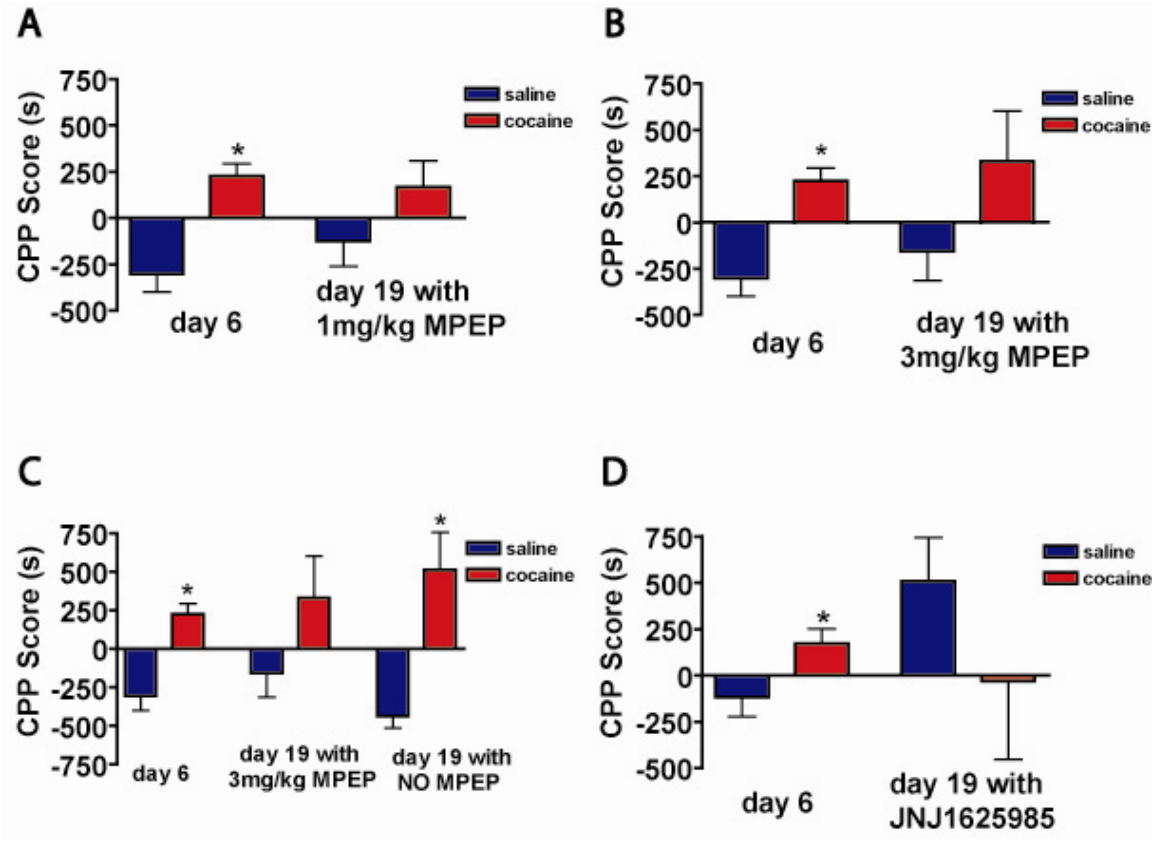

Figure 2. The effects of mGluR antagonists on cocaine-induced CPP. A. CPP was slightly reduced by pretreatment with 1mg/kg MPEP. Bar graph describes the CPP score for saline and cocaine treated rats. Cocaine treated rats showed significantly greater CPP scores on day 6 (n=24) but not on day 19 after 1mg/kg MPEP (n=7). B. Higher concentration of MPEP (3mg/kg) increased preference of saline treated animals for the non-preferred side. Cocaine treated rats showed significantly greater CPP scores on day 6 (n=24) but not on day 19 after 3mg/kg MPEP (n=5). C. Non-specific effects of MPEP were reversible. Cocaine treated rats showed significantly greater CPP scores on day 19 (n=5) when no MPEP was administered compared to saline treated rats (n=5). D. MGluR1 antagonist increased preference of saline treated animals for the non-preferred side. Cocaine treated rats showed significantly greater CPP scores compared to saline treated rats on day 6 (n=32) but not on day 19 after 2.5 mg/kg JNJ1625965 (n=8). *$p<0.05$.

Animals were conditioned with the CPP paradigm, and slices were prepared two weeks after the last injection. Using paired pulse stimulation DFB induced LTP (160.9 ± 12.7%, n=8) in the BLA-CeA pathway in slices from saline treated rats (**Fig 3B**). In slices from cocaine treated rats DFB also induced LTP (149.2 ± 15.7% compared to baseline, n= 8), and there was no significant difference between saline and cocaine treated rats (unpaired t test: t=0.580, p>0.05, n=8). The results in slices from cocaine (unpaired t test: t=0.365, p>0.05) and saline (unpaired t test: t=0.220, p>0.05) treated rats were similar compared to the LTP induced in drug naïve rats. These data indicated that the changes in mGluR5-mediated responses recorded in other brain areas after cocaine withdrawal are not applicable to this pathway in the amygdala.

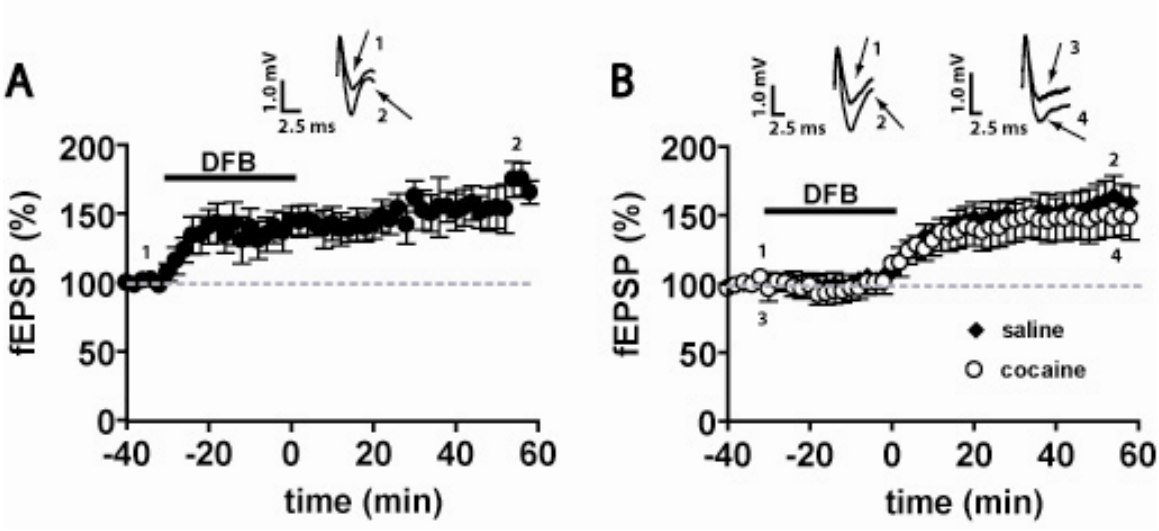

Figure 3. DFB induced LTP in the BLA-CeA pathway and its effect was unchanged after withdrawal from chronic cocaine. A. Graph depicts fEPSP slope (% of baseline) over time (min). Baseline fEPSPs were recorded for 10 min. Subsequently, 30μM DFB was applied for 30 minutes and fEPSPs were recorded for another 60 minutes. fEPSP slope values were significantly increased after DFB application (n= 6), indicating a long lasting potentiation. B. fEPSP slope values were significantly increased after DFB application in saline and cocaine treated rats (n=8).

A moderate DHPG dose induced LTP in the BLA-CeA pathway

To determine whether the lack of response to DFB after chronic cocaine was due to its unique site of action we initially tested DHPG, the mGluR1 and mGluR5 agonist, applied 15 minutes on drug at three different concentrations (100nM, 10μM, and 100μM) in naïve rat brain slices using single pulse stimulation in the BLA-CeA pathway (**Fig. 4**). At the lowest concentration (100nM), no LTP was recorded (100.3 ± 11.9% compared to baseline, t=0.029, p>0.05, n=4). Similarly, at the highest concentration (100μM) no LTP was measured (107.4 ± 4.5%, t=1.660, p>0.05, n=4) but rather, a small short duration inhibitory effect was observed during DHPG application. However, DHPG at a moderate concentration (10μM) induced LTP (155.1 ± 14.7% compared to baseline, n=6) in the BLA-CeA pathway. The moderate concentration of DHPG was used in all subsequent experiments.

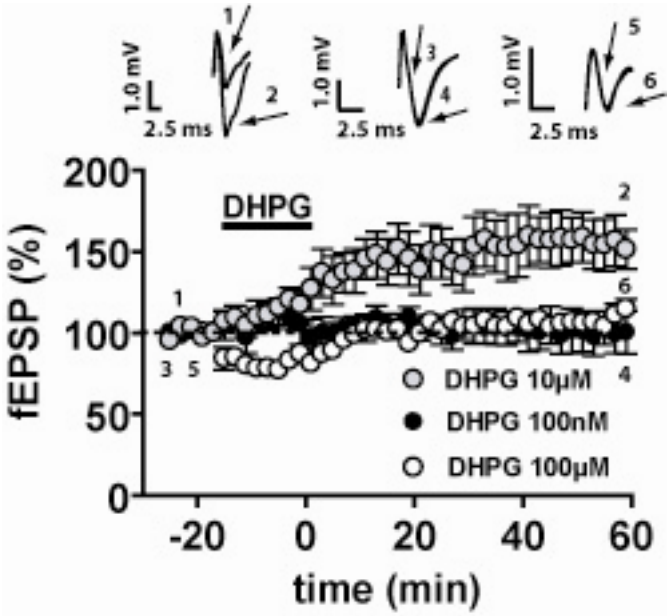

Figure 4. DHPG induced LTP at the 10 μM concentration. Baseline fEPSPs were recorded for 10 min. Following baseline recordings, DHPG was applied for 15 minutes followed by 60 minutes post-drug application recording. fEPSPs increased in slices receiving 10μM DHPG (n= 6) but not in slices receiving 100nM DHPG (n=4) or 100μM DHPG (n=4).

DHPG induced LTP through activation of mGlu5 receptors in the BLA-CeA pathway in slices from saline and cocaine treated rats

To further investigate mGluR5-mediated LTP, LY367385 (100μM), the selective mGluR1 antagonist, was applied for 25 minutes with (DHPG) (10μM) added to the solution during the last 15 minutes; this treatment paradigm effectively activated only mGlu5 receptors. Our results in slices from saline treated (135.7 ± 10.8%, n=9) and cocaine treated rats (128.3 ± 9.0% compared to baseline, n=9) showed that DHPG/LY367385 induced LTP using single pulse stimulation in the BLA-CeA pathway by the selective activation of mGluR5 (**Fig 5)** but that LTP recorded in slices from cocaine and saline treated animals did not differ significantly (unpaired t test, t=0.520, p>0.05, n=9). This finding was consistent with the DFB results and suggested that mGlu5 receptors were not altered after withdrawal from cocaine. We next examined potential changes in response to mGluR1 activation.

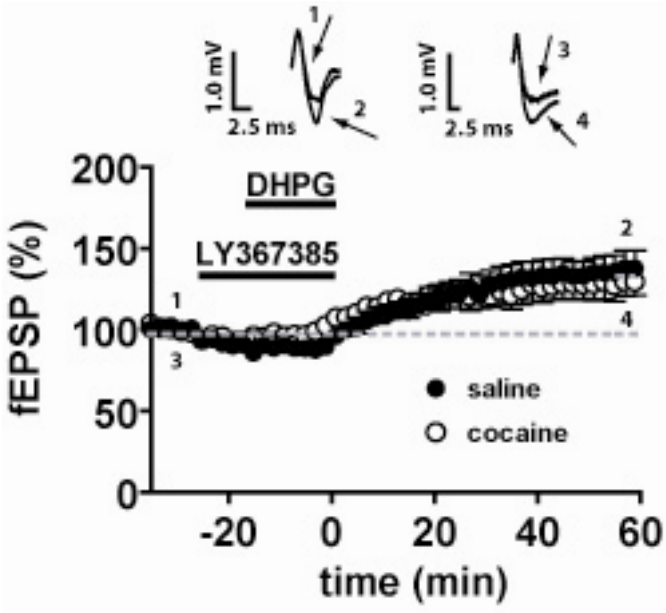

Figure 5. Selective activation of mGluR5 induced LTP in the BLA-CeA pathway. 100μM LY336385 was applied for 25 minutes with 10 μM DHPG added to the solution during the last 15 min followed by a post-drug application recording of 60 minutes. fEPSPs were significantly potentiated after DHPG application (n= 9) reflecting a mGluR5-mediated LTP.

MGluR1-mediated LTP was smaller in slices from cocaine treated rats

In these experiments MPEP (10μM) was applied for 25 minutes with DHPG (10μM) added to the solution during the last 15 minutes which activated only mGluR1. Using single pulse stimulation, DHPG/MPEP induced LTP (141.4 ± 8.9%, n=8) in the BLA-CeA pathway in slices from saline treated rats (**Fig 6**). In contrast, DHPG/MPEP induced a smaller LTP in slices from cocaine treated rats (115.5 ± 4.5%, n=8), and unmasked a small inhibitory effect on fEPSPs. There was a significant difference in LTP magnitude between saline and cocaine treated groups (unpaired t test: $t= 2.589$, $p<0.05$) indicating a change in mGluR1 responsiveness two weeks after cocaine treatment. To further investigate this change we examined pharmacological characteristics and downstream signaling molecules of the DHPG/MPEP-induced LTP.

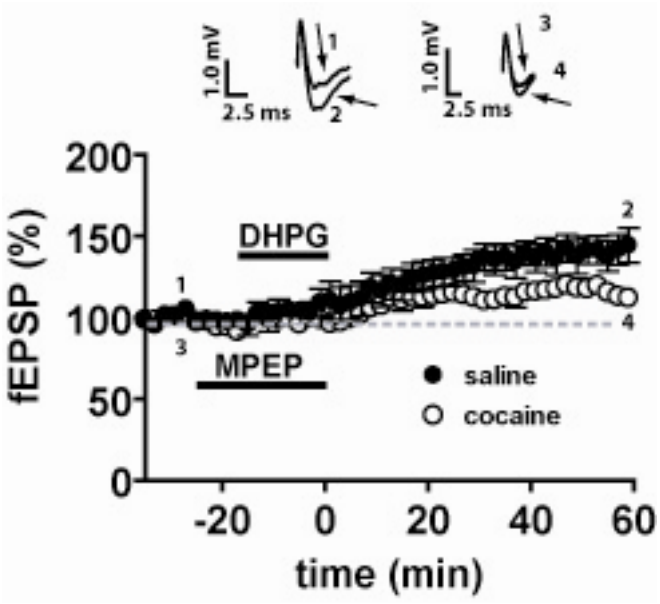

Figure 6. MGluR1 activation induced LTP in slices from saline treated rats which was reduced in slices from cocaine treated rats. 10μM MPEP was applied for 25 minutes with 10 μM DHPG added to the solution during the last 15 min. fEPSP slope increased in saline treated rats (n= 8) but LTP magnitude was significantly reduced in slices from cocaine treated rats (n=8).

MGluR1-mediated LTP was not dependent on NMDA receptors

We examined the role of NMDA receptors in mGluR1-induced LTP using the specific NMDA antagonist APV. APV (50μM) was applied for 25 minutes with DHPG (10μM) added to the solution during the last 15 minutes. In the presence of APV (50μM) mGluR1 activation also induced LTP (140.1 ± 13.8%, n=7), which was not significantly different from saline control slices (141.4 ± 8.9%, n=8; unpaired t test: t=0.077, p>0.05). These results suggested that mGluR1-mediated LTP in the BLA-CeA pathway was not dependent on NMDA receptors.

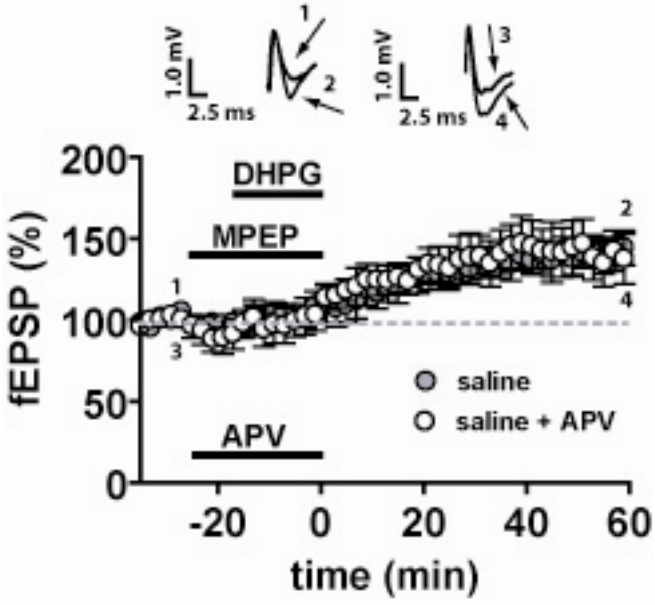

Figure7. MGluR1-mediated LTP was not dependent on NMDA receptors. 50μM APV was applied for 25 minutes. fEPSP slope increased in control slices (n= 8) and in APV treated slices (n=7) but there was no significant difference in the two curves.

The PKC inhibitor bisindolylmaleimide I (BIS-I) enhanced mGluR1-mediated LTP in the BLA-CeA pathway in slices from both cocaine and saline treated rats

When stimulated, group I mGluRs activate phospholipase C (PLC), which in turn increases the levels of diacyl glycerol (DAG) and PKC. Previous work has shown that DHPG-induced LTP in the rat dentate gyrus was prevented by PKC inhibitors (O'Leary and O'Connor, 1999). Our results in slices from both saline (217.2 ± 29.3%, n=4) and cocaine (194.4 ± 19.5%, n=6) treated rats show that mGluR1-mediated LTP was

significantly enhanced by the PKC inhibitor BIS-1 (1.2μM, **Fig. 8A, 8B**) relative to slices from control saline treated (141.4 ± 8.9%, n=8) (unpaired t test: $t=3.219$, $p<0.05$) and control cocaine treated rats (115.5 ± 4.5%, n=8) (unpaired t test: $t=4.512$, $p<0.05$). There was no significant difference between BIS-I treated slices from saline and cocaine animals (unpaired t test: $t=0.679$, $p>0.05$). These data suggested that PKC normally functions to tonically inhibit LTP in the BLA-CeA pathway and that after withdrawal from chronic cocaine removal of this tonic PKC inhibition returned synaptic plasticity to saline control levels suggesting enhanced PKC signaling after chronic cocaine withdrawal.

PKC isoforms are divided into typical and atypical families. Typical PKC isoforms are activated by the PLC-DAG pathway, whereas atypical PKC isoforms are not activated by PLC-DAG (Hopf et al., 2005). To further explore the PKC isoforms involved in the tonic inhibition of LTP, we next tested the effects of the PLC inhibitor, U73122, on LTP since PLC is upstream of PKC. Our results in slices from cocaine treated rats showed mGluR1-mediated LTP compared to baseline (177.2 ± 11.4%, n=5) was significantly enhanced by the PLC inhibitor U73122 (1.0μM, **Fig. 8C**) compared to control slices from cocaine treated rats (115.5 ± 4.5%, n=8) (unpaired t test: $t=5.899$, $p<0.05$). There was no significant difference between BIS-I cocaine and U73122 cocaine treated slices (unpaired t test: $t=0.7182$, $p>0.05$). This result indicated that a typical PKC isoform contributed to the tonic inhibition of LTP because the result was similar to the data from the PKC inhibitor experiment. In contrast, in saline treated rats mGluR1-mediated LTP induced in slices in the absence (141.4 ± 8.9%, n=8) and presence of U73122 (155.0 ± 11.9%, n=6) were not significantly different (unpaired t test: $t=0.945$, $p>0.05$) (**Fig. 8D**) suggesting that an atypical PKC contributed to the tonic inhibition of mGluR1-mediated LTP in saline treated animals. These data also indicated that the changes in the PLC-PKC pathway may be involved in the reduction of mGluR1-mediated LTP in slices from cocaine treated animals. Because atypical PKC can be activated by PKA (Huang et al., 2001), we next investigated the role of PKA in DHPG/MPEP-induced LTP.

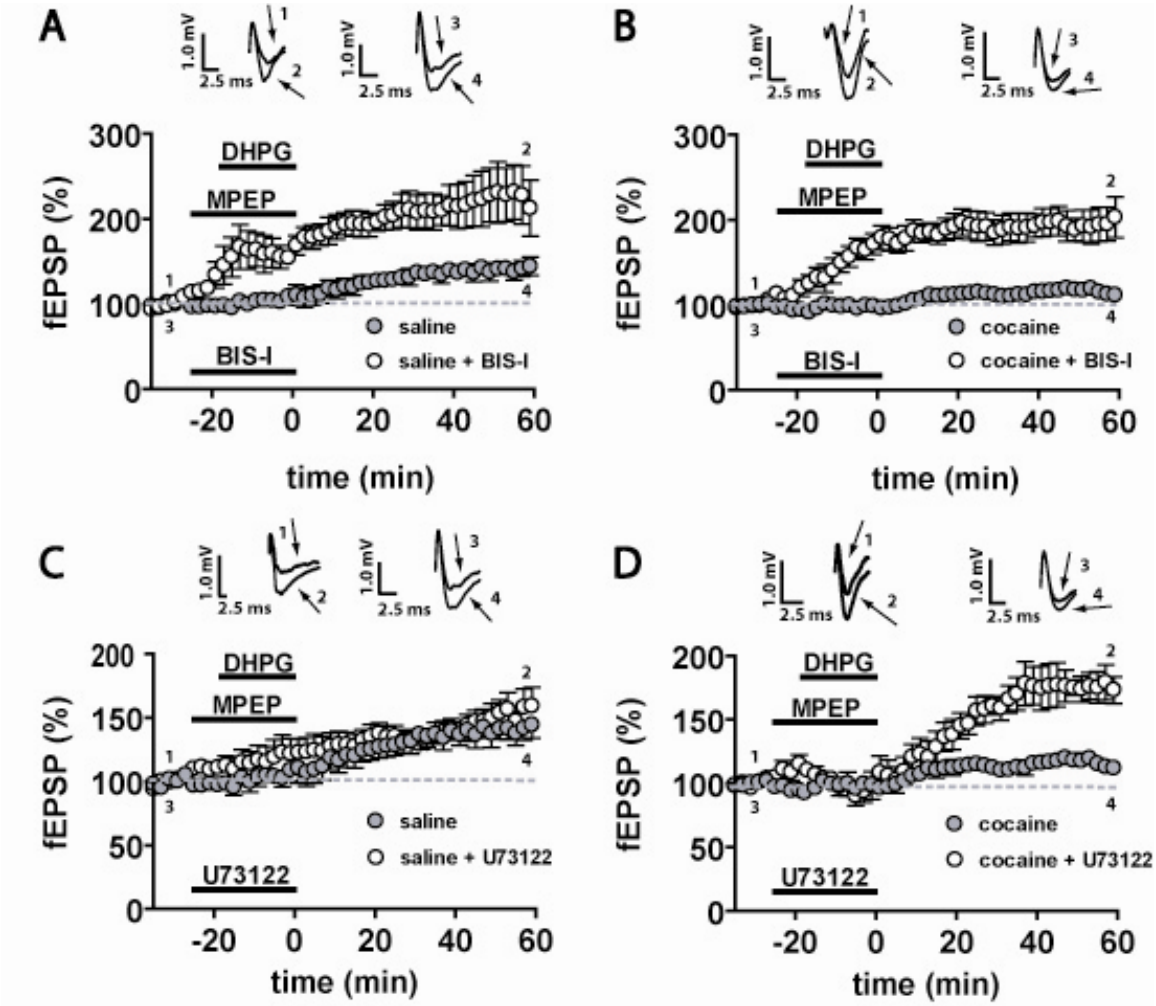

Figure 8. MGluR1-mediated LTP in the BLA-CeA pathway was enhanced by a PKC and a PLC inhibitor in chronic cocaine treated slices. 1.2μM BIS-I or 1.0 μM U73122 was applied for 25 minutes. A. fEPSP slope in BIS-I treated saline slices (n= 4) was significantly increased compared to saline control (n=8). B. fEPSP magnitude in BIS-I treated slices from cocaine treated animals (n= 6) was also increased compared to cocaine control slices (n=8). C. fEPSP slope values in U73122 treated cocaine rats (n= 5) compared to cocaine control rats (n=8). D. fEPSP slope values in U73122 treated saline rats (n= 6) and in saline control rats (n=8).

The PKA inhibitor H-89 enhanced mGluR1-mediated LTP in slices from cocaine but not saline treated rats

When activated, mGluRs linked to adenylyl cyclase stimulate PKA. It was shown that slow-onset LTP in the hippocampus was mediated by mGluR5 but not mGluR1 and was Ca^{2+} and PKA dependent (Lante et al., 2006). Our results showed that there was no significant difference in LTP magnitude in saline slices in the absence (141.4 ± 8.9%, n=8) (**Fig 9A**) and presence of the PKA inhibitor, H-89 (10μM) (143.0 ± 9.2%, n=5) (unpaired t test: t=0.120, p>0.05). These data suggested that PKA does not play a role in DHPG/MPEP-induced LTP in slices from saline treated rats. However, an atypical PKC isoform can still be responsible for tonic inhibition; this data only suggested that it was not activated by PKA. H-89 (10μM) treated slices from cocaine-treated animals showed a greater magnitude LTP (138.0 ± 12.3%, compared to baseline, n=6) compared to cocaine control slices (115.5 ± 4.5%, n=8) (one-way ANOVA: t=20.98, p<0.001, **Fig 9B**).

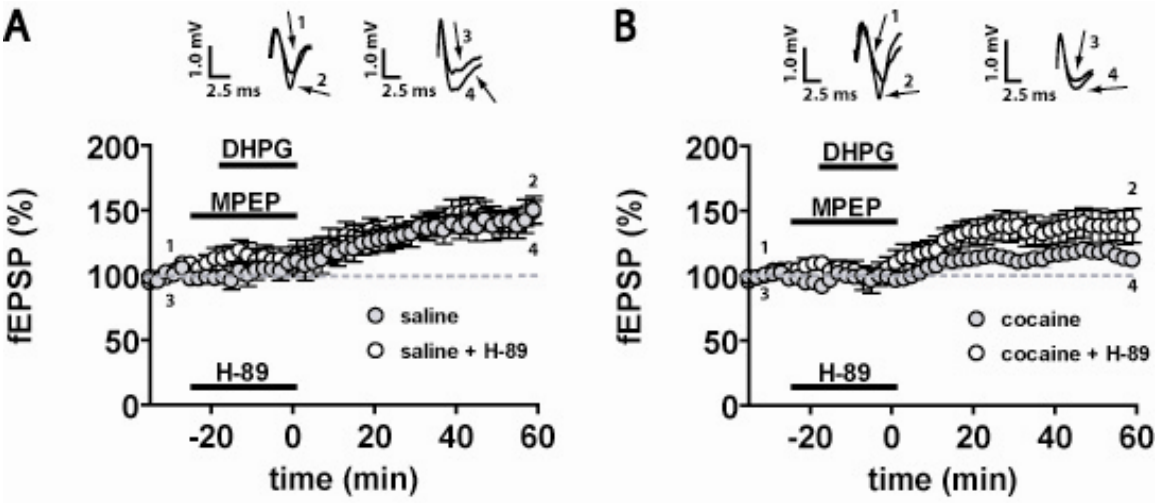

Figure 9. MGluR1-mediated LTP in the BLA-CeA pathway was enhanced by H-89 in slices from cocaine treated rats. 10μM H-89 was applied for 25 minutes. A. fEPSP slope values increased in saline treated control rats (n= 8) and in H-89 treated saline rats (n=5). B. fEPSP slope values increased in H-89 treated cocaine rats (n= 6) compared to cocaine control rats (n=8).

Additionally, there was no significant difference in LTP in the presence of H-89 in slices from saline and cocaine treated animals (unpaired t test: t=0.315, p>0.05). The results suggested that PKA functions to inhibit DHPG/MPEP-induced LTP after two weeks withdrawal from cocaine although increased PKA is present in the NAc and ventral tegmental area after 7 but not 21 days of withdrawal (Hope et al., 2005).

A MEK inhibitor had no effect on mGluR1-mediated LTP

Cocaine-induced craving is known to be mediated by ERK levels in the central amygdala (Lu et al., 2005). Since ERK formation is governed by MEK1/2 kinases, we compared DHPG/MPEP-induced LTP in the absence (141.4 ± 8.9%, n=8) and presence (140.0 ± 13.6%, n=7) of the MEK inhibitor PD98059 (50μM) (**Fig 10A**) in slices from saline treated animals. However, there was no significant difference (unpaired t test: t=0.086, p>0.05) in LTP magnitude indicating that mGluR1-mediated LTP was not dependent on MEK.

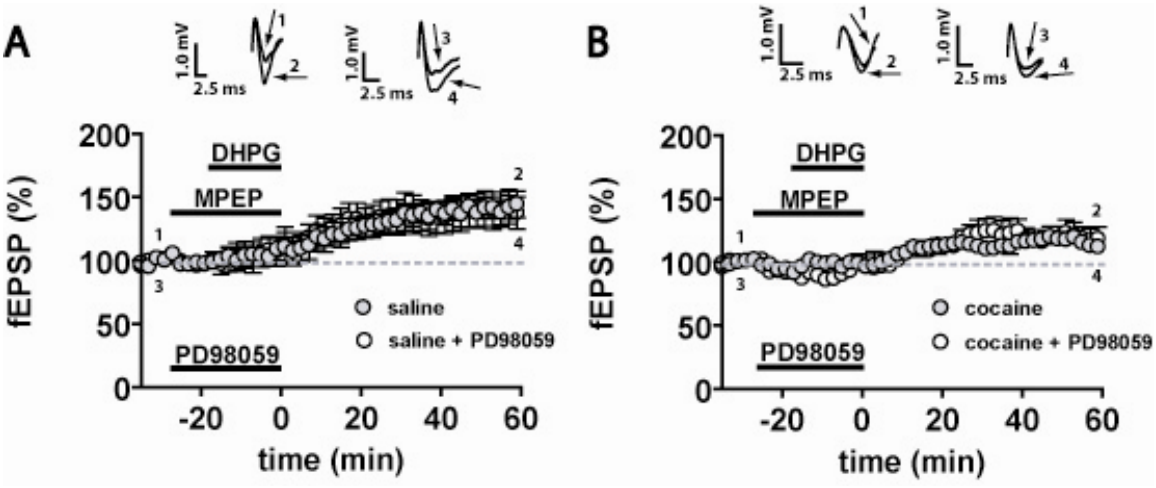

Figure 10. MGluR1-mediated LTP was independent of MEK. 50μM PD98059 was applied for 25 minutes. A. fEPSP slope values increased in saline treated control rats (n= 8) and in MEK treated saline rats (n=7). B. fEPSP slope values slightly increased in cocaine treated control rats (n= 8) and in MEK treated cocaine rats (n=5).

In slices from animals treated with cocaine and withdrawn for two weeks, there was also no significant difference (unpaired t test: $t=0.410$, $p>0.05$) in fEPSPs in the absence ($115.5 \pm 4.5\%$, $n=8$) and presence of the MEK inhibitor ($116.6 \pm 11.2\%$, $n=5$) suggesting MEK also did not play a role in mGluR1-mediated LTP after two weeks withdrawal from cocaine (**Fig 10B**).

Endocannabinoids mediate the mGluR1-mediated LTP in the BLA to CeA pathway

It has been shown that group I mGluR activation triggers the release of endocannabinoids (eCBs) (Wilson and Nicoll, 2002). Several groups have studied the essential role of eCBs in synaptic plasticity. One group found that eCBs can facilitate the induction of LTP in the CA1 region (Carlson et al., 2002). We examined the role of CB1 receptors in mGluR1-mediated LTP using the specific CB1 antagonist, AM251 (1μM). In slices from saline treated animals, DHPG/MPEP-induced LTP ($141.4 \pm 8.9\%$, $n=8$) was reduced ($118.2 \pm 6.4\%$, $n= 8$) significantly (one-way ANOVA: $t=22.69$, $p<0.001$) in the presence of AM251 (**Fig 11A**). In slices from animals treated with cocaine and withdrawn for two weeks, fEPSP slopes in the presence ($115.6 \pm 8.4\%$, $n= 8$) or the absence ($115.5 \pm 4.5\%$, $n=8$) of AM251 were not significantly different (one-way ANOVA: $t=2.366$, $p>0.05$) (**Fig 11B**). Additionally, there was no significant difference between AM251 slices from saline treated animals and control slices from cocaine treated animals (unpaired t test: $t=0.344$, $p>0.05$). These data suggested that the reduction in DHPG/MPEP-induced LTP in cocaine may be due to loss of an endocannabinoid contribution.

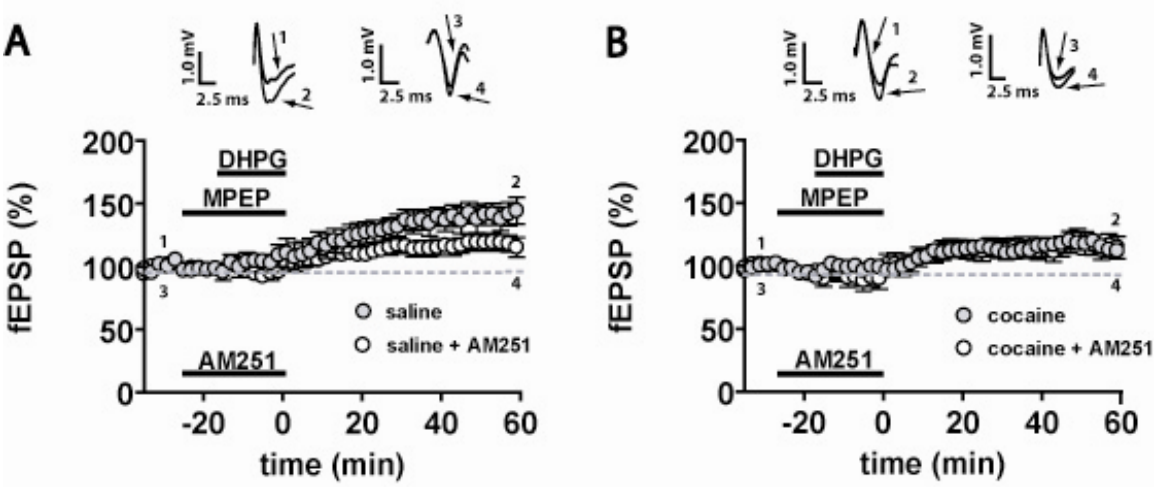

Figure 11. DHPG/MPEP-induced LTP via mGluR1 in slices from saline treated rats was dependent on CB1 receptor activation. 1μM AM251 was applied for 25 minutes. A. Blocking CB1 significantly reduced mGluR1-mediated LTP in saline treated rats (n=8). B. Blocking CB1 had no effect on LTP recorded in slices from cocaine treated rats (n=8).

Change in mGluR1-mediated LTP was not a function of cocaine withdrawal time

To investigate the effect of DHPG/MPEP-induced LTP in acutely withdrawn cocaine treated animals, slices were prepared from CPP trained animals 48 hours after the last injection. DHPG/MPEP induced LTP (115.1 ± 4.6% compared to baseline, n=5) in slices from acutely withdrawn cocaine treated rats and from cocaine treated rats withdrawn for two weeks (115.5 ± 4.5%, n=8) (**Fig 12B**). There was no significant difference in LTP magnitude between acutely and chronically withdrawn cocaine treated groups (unpaired t test: $t=0.059$, $p>0.05$). DHPG/MPEP also induced LTP compared to baseline in slices from acutely (143.1 ± 11.5%, n=6) and chronically (141.4 ± 8.9%, n=8) withdrawn saline treated animals (**Fig 12A**). Similarly, there was no significant difference in LTP magnitude between the acutely withdrawn and chronically withdrawn saline treated groups (unpaired t test: $t=0.118$, $p>0.05$). These data suggested that the change in mGluR1 responsiveness after cocaine treatment was due to the treatment itself and not the length of the withdrawal period.

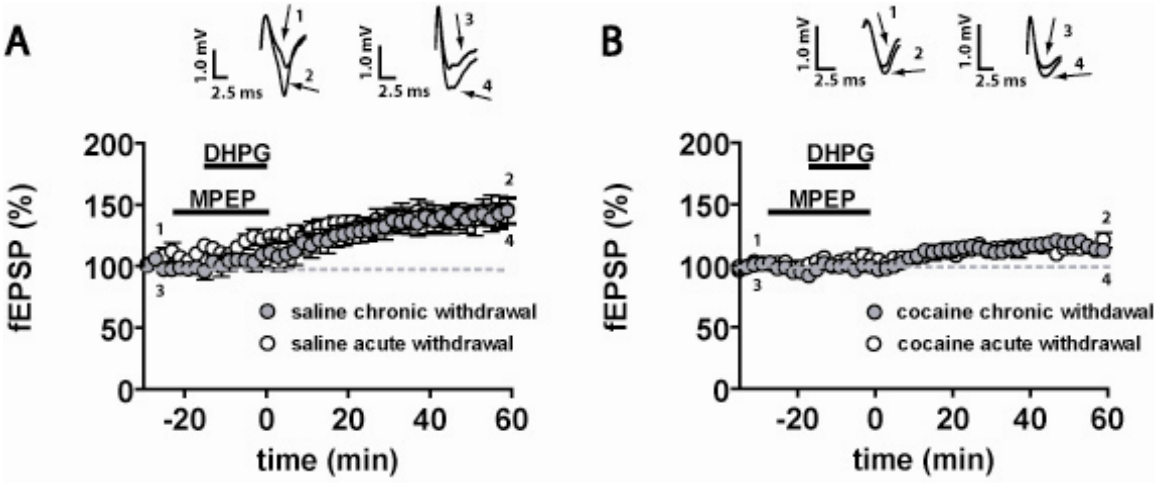

Figure 12. The reduced mGluR1-mediated LTP in slices from cocaine treated rats was not dependent on withdrawal time. A. Increase in fEPSP slope values in acutely withdrawn saline treated rats (n= 6) was equivalent to that from chronically withdrawn saline treated rats (n=8). B. fEPSP slope values slightly increased in acutely withdrawn cocaine treated rats (n=5) and chronically withdrawn cocaine treated rats (n=8).

Differences between mGluR1-mediated LTP in slices from saline and cocaine treated animals were abolished by blocking GABA receptors

It is possible that LTP in the BLA-CeA pathway was partially due to GABA inhibition. To investigate this we applied a high concentration of a GABA receptor inhibitor, picrotoxin (PTX) (50μM) to slices from naïve animals. In a higher concentration of PTX, mGluR1-mediated LTP (124.7 ± 5.1%, n=8) was slightly reduced compared to slices from control naive treated animals (134.5 ± 3.9%, n=5) that received 10 μM PTX (**Fig 13A**), but the difference was not significant (unpaired t test: t=1.343 p>0.05).

We next repeated this experiment in slices from animals treated with cocaine and saline. In the presence of DHPG/MPEP and 50μM PTX slices from saline treated animals showed LTP compared to baseline (136.9 ± 9.6%, n=6) during co-application of DHPG/MPEP and 50μM PTX that was not significantly different from slices receiving 10μM PTX (141.4 ± 8.9%, n=8) (unpaired t test: t=0.335, p>0.05) (**Fig 13B**). Slices from cocaine treated animals also showed LTP compared to baseline (126.8 ± 7.4%, n=8).

Although the LTP magnitude increased in the higher PTX concentration, it was not significantly different from cocaine slices receiving 10μM PTX (115.5 ± 4.5%, n=8) (unpaired t test: t=1.301, p>0.05) (**Fig 13C**). However, LTP magnitude in slices from cocaine treated animals (50μM PTX) (126.8 ± 7.4%, n=8) was not significantly different than in slices from saline treated animals (50μM PTX) (136.9 ± 9.6%, n=6) (unpaired t test, t=0.852, p>0.05) (**Fig 13D**). These results showed that although increasing PTX did not significantly affect the magnitude of LTP in the different animal populations, completely blocking GABA inhibition eliminated the difference in magnitude of DHPG/MPEP-induced LTP in slices from saline and cocaine animals. These data suggested that the reduced LTP in slices from cocaine treated animals was dependent on the presence of GABA inhibition since when GABA inhibition was completely blocked, the difference usually seen between saline and cocaine treated animals (in 10μM PTX) was no longer present.

MGluR1-mediated LTP was dependent on AMPA receptors

DHPG-induced plasticity is known to be dependent on NMDA receptors at some synapses and on AMPA receptors in others. To address this question we investigated the role of AMPA receptors in DHPG/MPEP-induced LTP. To slices from drug naïve animals we applied PTX (50μM) and the AMPA receptor antagonist, NBQX (10μM). Our results showed that compared to naive controls (124.7 ± 5.1%, n=8), the mGluR1-mediated LTP (112.4 ± 4.0, n=6) was reduced even further when AMPA receptors were blocked indicating that contribution of NMDA receptor mediated LTP was very small (**Fig 14**) (unpaired t test: t=1.778, p>0.05). This finding was consistent with our previous experiment in which the NMDA receptor antagonist, APV, had no effect on LTP in slices from saline treated animals.

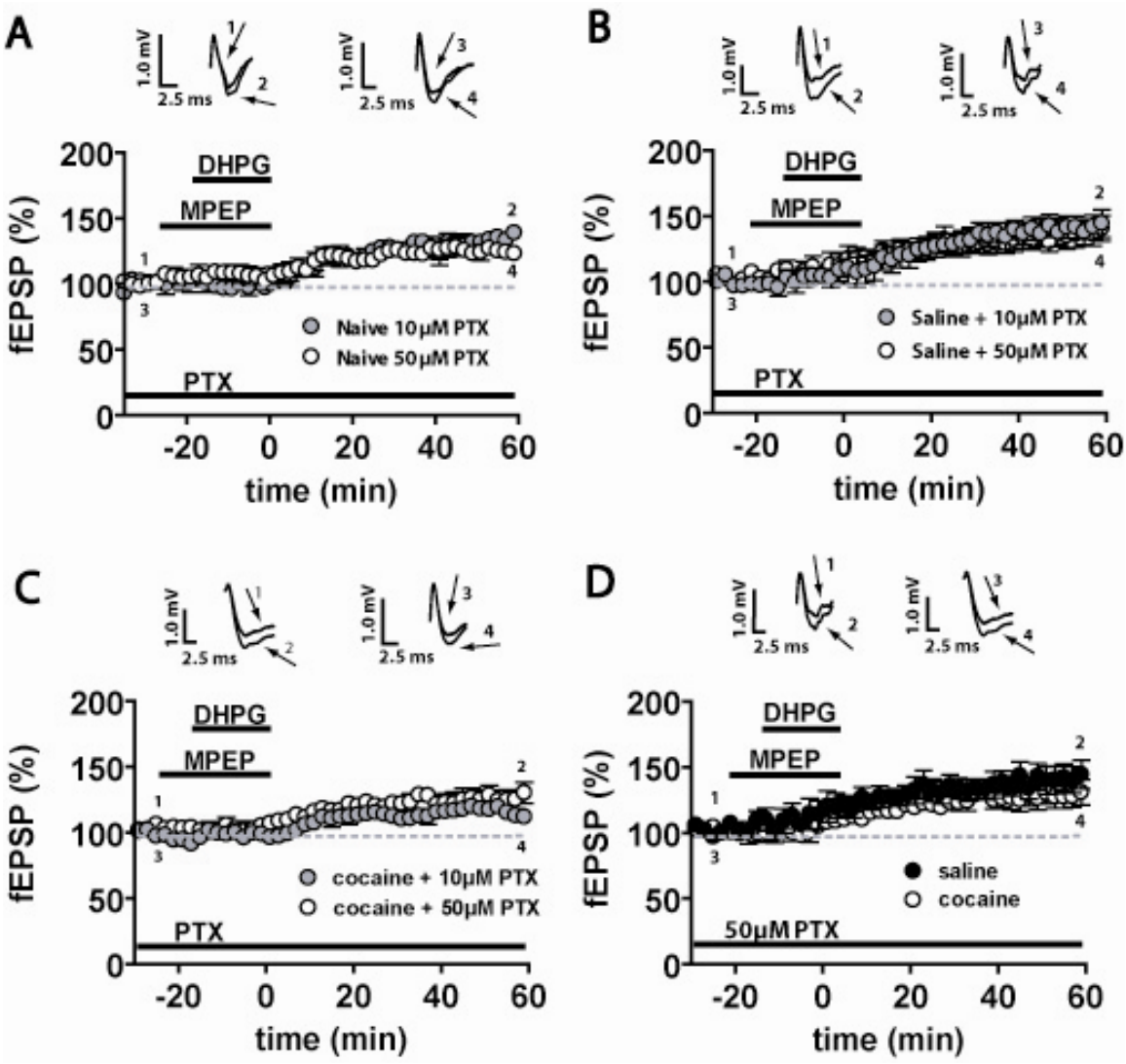

Figure 13. The effects of blocking GABA receptors on mGluR1-mediated LTP. 10μM or 50μM PTX was applied during the entire recording. A. Blocking GABA receptors did not affect mGluR1-mediated LTP in naive rats (n=8). B. Blocking GABA receptors did not change mGluR1-mediated LTP in saline treated rats (n=6). C. Blocking GABA receptors did not change mGluR1-mediated LTP in cocaine treated rats (n=8). D. fEPSP slope values increased in saline (n=6) and cocaine (n=8) rats in a high concentration of PTX.

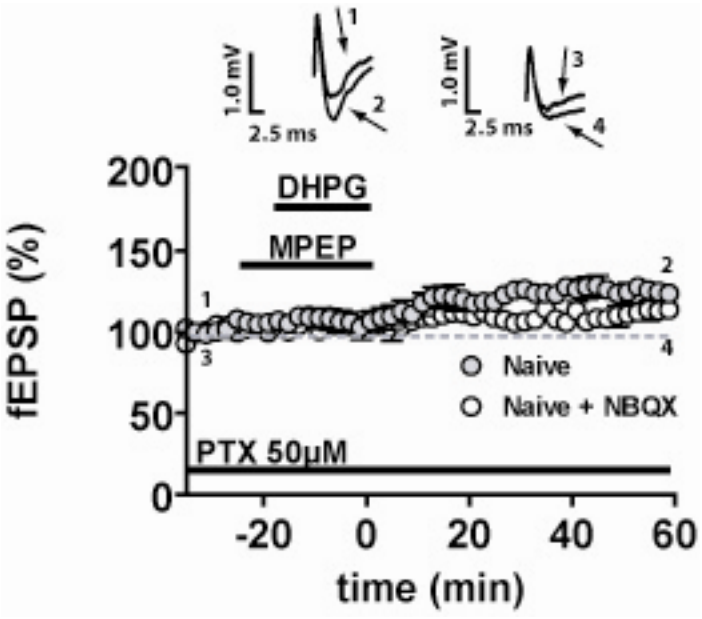

Figure 14. MGluR1-mediated LTP was reduced by blocking AMPA receptors. In the presence of 50 µM PTX and 10 µM NBQX, 10µM MPEP was applied for 25 minutes with DHPG added to the solution during the last 15 minutes. Blocking AMPA receptors reduced LTP in naïve rats (n=8).

DISCUSSION

The main findings from our studies are: (1) MPEP and JNJ16259685 reduced place preference in all treatment groups indicating a non-specific anxiolytic effect when injected intraperitoneally. (2) MGluR5-mediated LTP in the BLA-CeA pathway was not affected by chronic cocaine withdrawal. (3) MGluR1-mediated LTP in the BLA-CeA pathway was reduced after both acute and chronic withdrawal from cocaine. (4) MGluR1-mediated LTP was tonically reduced by PKC in slices from saline and cocaine treated animals and by PKA in slices from cocaine treated animals (5) MGluR1-mediated LTP was not dependent on MEK. (6) MGluR1-mediated LTP was enhanced by endocannabinoids in saline but not cocaine treated groups. (7) MGluR1-mediated LTP was AMPA dependent, NMDA independent and was partially due to GABA inhibition.

MPEP and JNJ16259685 reduced place preference in all treatment groups indicating a non-specific anxiolytic effect when injected intraperitoneally

When exposed to the two-sided conditioning chamber under low light conditions, our animals showed a natural preference for the black side indicating our CPP apparatus was a biased design. A biased design allows a larger scale for detecting time differences than a 50/50 distribution of time in an unbiased design (Roma and Riley, 2005). After 5 exposures to the conditioning chamber, our animals showed conditioned place preference for the drug paired side that persisted for at least two weeks.

Metabotropic glutamate receptors have previously been implicated in the expression of CPP. We found that CPP was slightly reduced with pretreatment of 1mg/kg of MPEP on test day 19; however, our results were not significant. Other groups have found significant reduction in CPP when injecting mGluR antagonists. MPEP dose-dependently reduced the development of cocaine-induced CPP (McGeehan and Olive, 2003) and the acquisition and expression of morphine-induced CPP in mice (Popik and Wrobel, 2002; Aoki et al., 2004). However, in rats only a high dose (50mg/kg i.p) of MPEP blocked morphine-, but not cocaine-induced CPP, and that the lower dose (10mg/kg i.p.) was not effective in either paradigm (Herzig and Schmidt, 2004).

We subsequently tested a higher dose of MPEP (3mg/kg) on test day 19 but found that this dose reduced place preference in both saline and cocaine animals, indicating that intraperitoneally injected MPEP had a non-specific effect. We then investigated the effects of JNJ16259685 on CPP to explore the role of mGluR1. Again, both cocaine and saline treated animals showed reduced preference for either side of the chamber. MGluR1 antagonists act as anxiolytics (Ballard et al., 2005), and decreasing anxiety may have caused a lack of discrimination of sides as shown in a previous study (Perez de la Mora et al., 2006). Future studies should be conducted on the role of mGluR5 and mGluR1 on cocaine-induced CPP with the antagonists injected directly into the amygdala to avoid the nonspecific effects seen when given intraperitoneally.

MGluR5-mediated LTP in the BLA-CeA pathway was not affected by withdrawal from chronic cocaine

MGluR5-mediated LTP in the BLA-CeA pathway was not altered after chronic cocaine administration and two week withdrawal. We induced mGluR5-mediated LTP using two approaches: a positive allosteric modulator, DFB, and a Group I mGluR agonist, DHPG, in the presence of an mGluR1 antagonist, LY367385. In both experiments there was no significant difference in LTP magnitude between slices from saline and cocaine treated animals. This suggested that changes in mGluR5-mediated responses seen in other brain regions after cocaine administration and withdrawal are not applicable to the amygdala.

It has been previously demonstrated that DHPG showed reduced capacity to induce glutamate release in the NAc after repeated cocaine administration (Swanson et al., 2001). Our results suggested that mGluR5 activation induced enough glutamate to produce LTP in the BLA-CeA pathway after cocaine administration; alternatively, mGlu5 receptors may be upregulated after cocaine exposure as seen after 3 weeks of cocaine withdrawal in the NAc (Ghasemzadeh et al., 1999).

Our results also contradict the recent study that indicated mGluR5 as a major player in synaptic plasticity alterations resulting from cocaine administration in the bed nucleus of the stria terminalis (BNST). Grueter and co-workers (2006) demonstrated that

mGluR5-mediated LTD in the BNST was reduced after multiple exposures to cocaine in mice. In our studies in the rat amygdala, mGluR5 activation using a comparable dose (100μM) of DHPG induced neither LTP nor LTD. In our hands, a smaller dose of DHPG (10μM) induced LTP in the BLA-CeA pathway. Furthermore, mGluR5-mediated LTP in this synapse was not altered after two weeks withdrawal from chronic cocaine.

The persistence of mGluR5-mediated LTP after cocaine injections and withdrawal is supported by behavioral data. Herzig and Schmidt (2004) showed that neither a low (10mg/kg i.p.) nor a high (50mg/kg i.p) dose of the mGluR5 antagonist, MPEP, blocked cocaine-induced CPP. Additionally, in a recent study, the expression of behavioral sensitization to cocaine-induced locomotor activity was found to be reduced by pretreatment with an mGluR1 antagonist but not an mGluR5 antagonist (Dravolina et al., 2006).

MGluR1-mediated LTP in the BLA-CeA pathway was reduced after both acute and chronic withdrawal from cocaine

Activation of mGluR1 by DHPG in the presence of MPEP induced LTP in the rat BLA-CeA pathway. MGluR-LTD in the rat dentate gyrus was mediated mainly by mGluR5 but partially by mGluR1 (Wang et al., 2007). In contrast, the block of DHPG-induced LTD in the CA1 required inhibition of both mGluR1 and mGluR5 (Volk et al., 2006) indicating that activation of mGluR1 or mGluR5 alone can induce LTD. Similarly, we found that either mGluR1 or mGlu5 activation was sufficient to induce LTP. However, many other studies showed that mGluR5 was required for the activation of electrically induced LTP (Lee et al., 2002; Fendt and Schmidt, 2002; Rodrigues et al., 2002). The amygdala showed moderate expression of mGluR5 protein levels (Romano et al., 1995) and low expression of mGluR1 protein levels (Martin et al., 1992), both of which were adequate to induce LTP according to our results.

We demonstrated that after two week withdrawal from chronic cocaine DHPG/MPEP-induced LTP via was significantly reduced. When animals were sacrificed two days, rather than 14 days, after their last cocaine injection we saw a similar reduction

in LTP in slices from cocaine treated animals. These data suggested that the change in mGluR1-mediated LTP was not a function of cocaine withdrawal time.

The reduced LTP observed in slices from cocaine treated animals could have resulted from a decrease in the number of mGlu1 receptors, a change in downstream signaling molecules, or a change in the possible action of mGluR1 on GABA inhibition. Additionally, a decrease in the amount of glutamate released after cocaine administration (Swanson et al., 2001) could cause a decrease in mGluR1-mediated LTP despite having no impact on mGluR5-mediated LTP if, for example, mGluR5 receptors are predominantly extrasynaptic. We proceeded to further characterize DHPG/MPEP-induced LTP and to investigate the role of downstream signaling molecules as well as the role of GABA inhibition in mGluR1-mediated LTP.

MGluR1-mediated LTP was tonically reduced by PKC in slices from saline and cocaine treated animals and by PKA in slices from cocaine treated animals

The reduction in DHPG/MPEP-induced LTP measured in cocaine treated animals could have been caused by a change in downstream signaling molecules affected by cocaine. To investigate this we first explored the role of PKC in mGluR1-mediated LTP. In the presence of a PKC inhibitor, slices from both saline and cocaine treated animals showed enhanced DHPG/MPEP-mediated LTP magnitude. This contradicted other studies that have shown group I agonist-induced LTP was dependent on PKC in the rat dentate gyrus (O'Leary and O'Connor, 1999; Camodeca et al., 1999). However, our results were consistent with data on PKC's involvement in LTD. Activation of PKC was necessary for the induction of mGluR-LTD in the rat dentate gyrus (Wu et al., 2004), and HFS-LTD was also mediated by PKC in the dentate gyrus (Wang et al., 2007). Our results indicated that PKC normally functions to tonically inhibit LTP in the BLA-CeA pathway possibly in the same way it served to induce LTD at other synapses.

To corroborate our PKC results and further explore the PKC isoforms involved in mGluR1-medaited LTP we tested the effects of a PLC inhibitor on LTP. In slices from saline treated animals the LTP in the presence of the PLC inhibitor was not different than controls. It also was not statistically different from the PKC-inhibited LTP in the saline

treated group. Liang and co-workers (2005) found that mGluR-induced LTP requires activation of PLC in trigeminal synapses, but our data indicated that the PLC-PKC pathway served to reduce LTP in the amygdala. These results suggested that an atypical PKC (Hopf et al., 2005) may have been involved in the tonic inhibition of mGluR1-mediated LTP in saline treated animals, because the atypical PKC isoform does not require activation by DAG or PLC. In contrast, in slices from cocaine treated animals we saw enhanced LTP suggesting that a typical PKC seems to be recruited with cocaine treated animals. The results indicated that the changes in the PLC-PKC pathway may be involved in the reduction of mGluR1-mediated LTP.

Because atypical PKC can be activated by PKA (Huang et al., 2001) we investigated the role of PKA in mGluR1-mediated LTP. In slices from saline treated animals there was no change in LTP magnitude in the presence and absence of the PKA inhibitor. This suggested that PKA may not play a role in DHPG/MPEP-induced LTP in saline treated animals although an atypical PKC isoform still contributed to the tonic inhibition of LTP. Similarly, the induction of LTD by DHPG in the dentate gyrus was not affected by a PKA inhibitor (Camodeca et al., 1999). In contrast, it was recently shown that slow-onset low-frequency stimulation-induced LTP in the hippocampus was PKA dependent (Lante et al., 2006). In our study, slices from animals treated with cocaine and withdrawn for two weeks showed a reduced LTP magnitude that was enhanced when PKA was blocked. This finding suggested that PKA, in addition to a typical PKC isoform, may play a tonic inhibitory role in mGluR-induced LTP only in the cocaine treated group. However, further experiments are needed to determine if PKA inhibitors can reduce the enhancement of LTP in the presence of PLC inhibitors.

MGluR1-mediated LTP was not dependent on MEK

The MAPK signaling pathway is thought to play a role in neuronal plasticity (Imprey et al., 1999). One member of the MAPK family, ERK, is thought to have a role in cocaine addiction (for review, see Lu et al., 2006). Because ERK is activated by MEK, we tested the effects a MEK inhibitor on mGluR1-mediated LTP. We found that

the LTP in slices from both saline and cocaine treated animals was not dependent on MEK.

Contrary to our results, recent studies have shown that ERK regulated mGluR-LTD in the BNST (Grueter et al., 2006), and in CA1 neurons (Gallagher et al., 2004). Additionally, a p38 MAPK inhibitor blocked DHPG-LTD in the dentate gyrus (Rush et al., 2002) and CA1 neurons (Huang et al., 2004). A MEK inhibitor also blocked DHPG-LTP in the rat dentate gyrus (Coogan et al., 1999). However, no previous electrophysiology studies in the basolateral-central amygdala pathway have been performed to assess the role of MEK in LTP or LTD after cocaine withdrawal. Here we present the novel finding that MEK was not required for LTP in the BLA-CeA pathway and was not altered by cocaine. In a recent review, Lu (2006) explains that MEK inhibitors injected into the central amygdala had no effect on cocaine self-administration suggesting that ERK activity in this brain region was not involved in drug reward as measured by self-administration, a concept supported by our results.

MGluR1-mediated LTP was enhanced by endocannabinoids in saline but not cocaine treated groups

MGluR1-mediated LTP in the BLA-CeA pathway was reduced in the presence of a CB1 antagonist indicating that eCBs normally function to enhance LTP at this synapse. It is hypothesized that Group I mGluR activation triggers release of eCBs that then retrogradely travel to the presynaptic neuron causing suppression of neurotransmitter release (Wilson and Nicoll, 2002). Additionally, several studies have found a role for eCBs in synaptic plasticity. For example, mGluR-LTD was mediated by eCBs (Robbe et al., 2002; Chevaleyre and Castillo, 2003) in various brain regions including the amygdala (Marsicano et al., 2002). One study found increased levels of anandamide, the CB1 agonist, during postnatal development that accounted for a switch from LTP to LTD in the striatum (Ade et al., 2007). Another group demonstrated that eCBs facilitated the induction of LTP in the CA1 region (Carlson et al., 2002).

Interestingly, other studies have shown that activating mGluR5 or mGluR1 and 5 together caused the release of eCBs in the hippocampus (Ohno-Shosaku et al., 2002;

Chevaleyre and Castillo, 2003) and the NAc (Robbe et al., 2002). In the BLA, however, it has been shown that endocannabinoid-mediated LTD required activation of primarily mGluR1 (Azad et al., 2004). Our results showed that DHPG can induce LTP via mGluR1 or mGluR5; however, cocaine-mediated changes in LTP were only recorded with mGluR1-mediated LTP in the BLA suggesting that changes in eCBs may be related to the reduced mGluR1-mediated LTP in the cocaine treated group.

The fact that DHPG/MPEP-induced LTP magnitude was reduced in the presence of a CB1 antagonist suggested that eCBs released upon mGluR1 activation were acting in a retrograde fashion on GABAergic neurons in the BLA-CeA pathway to reduce GABA release. Therefore, the reduction of mGluR1-mediated LTP measured in the cocaine treated group could be due to a loss in the endocannabinoid contribution.

Endogenous cannabinoids are involved in drug addiction (for review, see Maldonado et al., 2006) and cocaine exposure can alter the activity of eCBs. For example, a single cocaine exposure abolished eCB-LTD in the NAc (Fourgeaud et al., 2004). It is possible that the reduced DHPG/MPEP-induced LTP in cocaine treated animals was caused by altered eCBs. However, it was recently shown that cocaine self-administration did not alter the levels of eCBs in the NAc nor did a CB1 antagonist affect cocaine self-administration (Caille et al., 2007).

MGluR1-mediated LTP was AMPA dependent, NMDA independent and was partially due to GABA inhibition

MGluR1-mediated LTP in the BLA-CeA pathway was not dependent on NMDA receptors. Similar results were found in the dentate gyrus (O'Leary and O'Connor, 1997), the BNST (Grueter et al., 2006), and the CA1 region (Zho et al., 2002; Watabe et al., 2002). In contrast, DHPG-induced LTP was blocked by the NMDA antagonist AP5 in the medial vestibular nuclei (Grassi et al., 2002). To further investigate a NMDA component of DHPG/MPEP-induced LTP we applied an AMPA receptor antagonist, NBQX, to our solution and recorded LTP of small magnitude. Taken together these data suggested that mGluR1-mediated LTP was AMPA receptor dependent and NMDA receptor independent.

To determine the role of GABA inhibition in DHPG/MPEP-induced LTP we used a high concentration of PTX, a GABA receptor antagonist, in our solution. In the presence of a high concentration PTX the statistical difference between cocaine and saline LTP measured in low concentration PTX was eliminated. This finding suggested that the mechanism causing a reduced LTP in slices from cocaine treated animals may involve an upregulation of the GABA system.

Other studies have shown that DHPG differentially regulated GABA release in a variety of nuclei. Recently, one group showed that DHPG produced an increase in IPSCs in the thalamus (Govindaiah et al., 2006). Additionally, DHPG induced an outflow of GABA in the solitary nucleus (Jones et al., 1998) and an increase in GABA concentration in the periaqeuductal grey neurons (de Novellis et al., 2003). In the cerebellum, however, DHPG depressed GABAergic transmission (Galante et al., 2004). Likewise, inhibition of GABAergic transmission was recorded in periaqueductal grey neurons (Drew and Vaughan, 2004), the substantia nigra pars reticulate (Marino et al., 2001), and the CA1 region (Chevaleyre and Castillo, 2003). Another study revealed that in cortico-striatal slices DHPG reduced inhibitory currents that were blocked by a mGluR1 but not mGluR5 antagonist (Battaglia et al., 2001). Overall, DHPG affected GABA release in different ways depending on the brain region. Other studies have found that blocking GABA inhibition was required for electrically-induced LTP and that repeated cocaine treatment reduced GABA inhibition in midbrain dopamine neurons (Liu et al., 2005). A similar mechanism may underlie the reduced mGluR1-mediated LTP in the cocaine treated group in the BLA-CeA pathway where completely blocking GABA receptors increased DHPG/MPEP-induced LTP to almost saline control levels.

In conclusion, intraperitoneally injected Group I mGluR antagonists failed to block cocaine-induced CPP. Future studies should be conducted to directly inject the antagonists into the amygdala to avoid non-specific effects. Furthermore, chronic cocaine withdrawal did not affect mGluR5-mediated LTP in the BLA to CeA pathway; rather, mGluR1-mediated LTP was significantly reduced in slices from cocaine treated animals. This reduction was seen after both acute and chronic withdrawal from cocaine

and was AMPA receptor dependent and NMDA receptor independent. Additionally, mGluR1-mediated LTP was not dependent on MEK and was tonically reduced by an atypical PKC isoform in saline treated animals and by a typical PKC isoform in cocaine treated animals. MGluR1-mediated LTP was also tonically reduced by PKA but only in slices from cocaine treated animals. Finally, the mechanism of diminished mGluR1-mediated LTP after cocaine treatment may involve an upregulation in the GABA system and/or a disruption in endocannabinoid function.

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