Behavioral and Intracellular Mechanisms of mGluR5 within the Nucleus Acumbens Shell
during Expression of Environmental Elicited Cocaine Conditioning

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List of Abbreviations

(2R)-amino-5-phosphonovaleric acid (AP5)

(2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495)

(RS)-α-Cyclopropyl-4-phosphonophenylglycine (CPPG)

(RS)-α-Methyl-4-phosphonophenylglycine (MPPG)

(S)-3,5-Dihydroxyphenylglycine (DHPG)

2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP)

6-OHDA (6 Hydroxy dopamine)

7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOet)

Ambulatory Distance (AD)

AMPA Receptor Subunit GluR1 (GluA1)

Amygdala (Amyg)

Analysis of variance (ANOVA)

Calcium (Ca2+)

Calmodulin-dependent protein kinase II (CaMKII)

cAMP response element-binding protein (CREB)

Centimeter (cm)

Cocaine-paired (P)

Cocaine-unpaired (U)

Coiled-coil (CC)

Conditioning place preference (CPP)

Control (C)

Cyclic adenosine monophosphate (cAMP)

DA receptor 1 (DA1)

DA receptor 2 (DA2)

Day 1 (D1)

Day 10 (D10)

Day 12 (D12)
Day 5 (D5)

Diacylglycerol (DAG)

Dimethyl sulfoxide (DMSO)

Dopamine (DA)

Dopamine transporters (DAT)

Ena/VASP1 homology (EVH1)

Endoplasmic reticulum (ER)

Excitatory Amino Acid (EAA)

Excitatory amino acid transporters (EAAT)

Extracellular signal-regulated kinases (ERK)

Gamma-aminobutyric acid (GABA)

Glutamate receptors subtype 3 (GluR3)

Glutamate receptors subtype 4 (GluR4)

Hippocampus (HPC)

Intraperitoneal (ip)

Kainate (KA)

Knockout (KO)

\(L-(+)-2\text{-Amino-4-phosphonobutyric acid} (L-AP4)\)

Long Term Deppresion (LTD)

Long Term Pontentiation (LTP)

Mammalian Target of Rapamycin Complex 1 (mTORC1)

Mean±standard error of the mean (SEM)

Metabotropic glutamate receptors (mGluRs)

Mitogen- and stress-activated protein kinase 1 (MSK1)

Mitogen-activated protein kinases (MAPK)

National Institute of Abuse (NIDA)

N-Methyl-D-aspartate (NMDA)

Nucleus Accumbens (NAc)

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)
Phospholipase C/Inositol 1,4,5-triphosphate (PLC/IP3)
Prefrontal cortex (PFC)
Protein Kinase C gamma (PKCγ)
Sodium hydroxide (NaOH)
Striatum (Str)
Substantia Nigra (SN)
Threonine (Thr)
Total Move Time (TMT)
Total ERK (T-ERK)
Total pERK (T-pERK)
Transient receptor potential (TRP)
Tyrosine (Tyr)
Ventral pallidum (VP)
Ventral tegmental area (VTA)
Versus (vs.)
Vertical plane move time (VPT)
Vesicular glutamate transporters (VGLUT)
α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)
Abstract

Drug abuse is one of the major public health concerns affecting our society and an effective treatment for this pathology has not yet been developed. Addiction is defined as a chronic relapsing brain disease characterized by the compulsive use of drugs despite its harmful consequences (National Institute of Abuse (NIDA)). Drugs such as cocaine can cause addiction resulting in neurobiological and behavioral changes. These neurobiological changes make the person more susceptible to relapse after periods of abstinence. There are three main factors that can precipitate relapse in an addict; acute exposure to the drug, stress and environmental stimuli associated with previous drug use. It has been imperative and a challenge in the field of drug abuse to understand why environmental cues previously paired with drug use can elicit relapse after periods of abstinence. The present thesis seeks to contribute to the present knowledge related to the neurobiological substrates of cues-induced drug seeking behavior.

Addiction studies have identified different brain regions that are affected by drugs abuse, among them the nucleus accumbens (NAc). The NAc is localized within the ventral region of the corpus striatum (Str) and has been directly implicated in the mediation of psychostimulant-induced locomotor activity via dopamine (DA) and glutamate neurotransmission (Vezina & Kim, 1999). DA neurotransmission to the NAc is associated with reward and motivation, outputs from the NAc are associated with motor execution of goal-directed behavior (Kelley, 1999, Groenewegen, 1999), giving the NAc a dual function in the reward system. Based on its connections, the NAc have been divided in two subregions: the NAc shell and NAc core (Zahm & Brog, 1992). The shell has been implicated in the modulation of emotion while the core modulates the motor responses related to reward. A better understanding of the different genes or proteins within the NAc affected by cocaine exposure would help in identifying targets for specific pharmacotherapeutic clinical treatment. Appropriate targets may include Excitatory Amino Acid (EAA) receptors. EAA neurotransmission within the NAc helps to regulate the neural mechanisms underlying learning and
memory processes that register environmental stimuli promoting drug use. EAA exert their action through ionotropics and metabotropics receptors. There are eight metabotropic glutamate receptors (mGluRs) subtypes classified into three groups (Conn and Pin, 1997). All mGluRs groups are found in NAc subregions and have been implicated in mediating cocaine addiction, specifically mGluRs subtypes 1 and 5.

Group I mGluRs are associated to the Homer proteins, a family of synaptic proteins that anchor mGluR1/5 at the excitatory synapse and are implicated in the psychomotor effects of cocaine. Moreover, the Homer protein dependent-pathway has been linked to extracellular signal-regulated kinases (ERK) 1/2 and the subsequent activation of several transcription factors that may play a role in the associative learning involved in cocaine conditioning.

Even when there is extensive evidence that related mGluR5 in the modulation of different cocaine exposure paradigms, such as cocaine seeking behavior, and cocaine induced locomotion sensitization, there is not much evidence that can establish how mGluR5 within the NAc shell modulate environmental elicited cocaine locomotion conditioning. Moreover, it is not well known how development of locomotion conditioning affects mGluR5 and Homer1b/c protein expression within the NAc subregions. Thus, the present thesis further examines the role of mGluR5 within the NAc shell in the expression of environmental elicited cocaine conditioning. We were able to confirm the role of mGluR5 in cocaine conditioning paradigm. In addition, we established that mGluR5 and Homer 1b/c is not affected during acquisition neither expression of the conditioning. Furthermore, we believe that even when proteins expression of mGluR5 and Homer 1b/c is not affected their interaction is important for the downstream events that are activated and modulating the expression of the conditioning. Even when pERK1/2 was not affected in the later time of the expression of the conditioning, we assume that an early activation of ERK1/2 account for the later response of the conditioning.
Dedication

I want to dedicate this thesis to the many people that accompany me through this ‘journey’. First of all, I want to dedicate my thesis to my parents, Noemi Rivera “Mimi” y Angel L. Martínez “Luisito”. Thanks to them I achieved it!!! They were my support in all aspects, they encouraged me all these years, I really appreciate it, love you both. Mom you are such a huge warrior, you are my heroin, the rest of my life would not be enough to express you how thankful I am for all you have done, for your love and unconditional support. Dad, you also have been an essential part of my life and achievements, your unconditional love and kindness and all your support is indescribable, I will always thank you. Second, I dedicate it to my second “parents” Jackie y David they also gave me all the support that parents can give. To my brother, Angelito, for his supporting words. To my nephew Danielito, and to my cousins Jesús and Jarleen, you little guys mean a lot to me, thanks for your unconditional love to “Nina”.

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Behavioral and Intracellular Mechanisms of mGluR5 within the Nucleus Acumbens Shell during Expression of Environmental Elicited Cocaine Conditioning
Chapter I

General Introduction
Introduction

1. Drug Abuse

Drug abuse is characterized by the loss of control over drug use. This loss of control drives the person to develop addiction, a disease that affects the brain functionality and human behavior, resulting in an impact to society. Some consequences of drug abuse can result in thinking and memory problems, poor social behaviors, and bad performance at work. One of the major problems that face drug addicts is that after long periods of abstinence he/she will experience relapse repeatedly. There are three main factors that can elicit relapse; stress, acute exposure to the drug and environmental cues previously associated with drug exposure. Individuals may begin drug use by different reasons. First they will experience the pleasurable effects and they will think that they are in control, however, once the drug is abused it chronically “hijack” the brain reward circuitry by inhibiting the individual to control his/her drug intake. Even when the first intake of the drug by a person was probably voluntary, the continued craving for the drug results in a brain disease rather than a moral weakness. This disease is a consequence of the neurobiological changes created by the chronic use of the drug.

The mesocorticolimbic reward pathway (figure 1) is the crucial brain system that modulates pleasurable effects and the reinforcing effects of drugs of abuse. Natural rewards, such as food and sex, elicit a burst of DA release in the neurons of the mesocorticolimbic reward pathway resulting in the pleasurable and rewarding effects of these rewards. The same occurs when there is a drug of abuse in the system, but this time there is a higher response of DA signaling (Koob et al., 1994; Di Chiara, 1999; Parvaz et al., 2011). All drugs of abuse work in a direct or in an indirect manner to enhance DA levels in the NAc neurons. Drugs such as cocaine, methamphetamine, amphetamine and ecstasy promote an increase in DA levels by inhibiting DA reuptake or promoting its release through their effects on DA transporters (DAT) (Madras et al., 1989; Volkow and Ting-Kai, 2004). Other drugs, such as nicotine, alcohol, opiates and marijuana promote DA cell firing through the
stimulation of other neurons like Gamma-aminobutyric acid (GABA)-mediated or glutamatergic neurons (Kreek et al., 2002; Volkow and Ting-Kai, 2004). Although DA is the principal neurotransmitter driving the reinforcing and addictive effects of drugs of abuse there are other neurotransmitters, such as, glutamate, GABA, opioids, acetylcholine, cannabinoids and serotonin, within the reward circuit modulating these effects in an indirect manner (Boghdadi and Henning, 1997). The DA mesocorticolimbic pathway is composed of several brain regions, the main areas are the medial prefrontal cortex (mPFC), ventral tegmental area (VTA), the NAc, the amygdala (Amyg) and the hippocampus (HPC). The VTA is the one that contains the somas of the dopaminergic neurons that project to the NAc and to the mPFC. The NAc is in charge of regulating reward, while the mPFC modulates the goal directed-behavior, the Amyg processes the emotions triggered by drug taking, and the HPC mediates the learning and memory processes related to drug addiction.

The effects exerted by drugs within this circuitry are responsible for the development of addiction. Once a person continuously abuses a particular drug, this will elicit brain changes. For example, in order for the brain to create a balance for all the DA that has been released it starts to diminish the amounts of DA that are produced and also the receptors in the circuitry. As consequence, the person does not experiment pleasure in things that previously exerted that response. Another consequence of chronic (continuous and repeatedly) use of drugs is the loss of control and judgment. This loss of control in addition to other neurobiological modifications, including memory adaptations, leads to an enhanced motivational value of the drug at the expense of other reinforce that drives the person to an uncontrollable craving resulting in compulsive drug taking (Volkow et al., 2003, 2011).
Figure 1. Mesocorticolimbic reward pathway of the brain (Taken from Kauer and Malenka, 2007)
Adaptations that occur within the brain as consequence of chronic drug use are long lasting, making more difficult the development of an effective treatment for addiction. Moreover, the variety of circuits that are affected within the mesocorticolimbic pathway leads to the necessity of developing multimodal treatments. Furthermore, the unpleasant feelings of the withdrawal symptoms (such as dysphoria, depression, irritability, and anxiety among others) produced by the cessation of drug use, represent a challenge because even when all drugs work in a direct or in an indirect manner in the enhancement of DA levels, withdrawal symptoms differ among them.

2. Cocaine; an Addictive Psychostimulant

Cocaine is an addictive psychostimulant extracted from the leave of the coca plant *Erythroxylon coca*, a plant that is native from Peru and the western part of South America. Cocaine use dates from thousand years in were the Andean Indians from Perú, chewed the coca leaves in order to reduce symptoms associated with living at high altitudes such as dizziness (Karch, 1999). Cocaine leaves were also chewed by the Indians because of their euphorigenic properties, the ability to reduce fatigue and hunger, and to increase stamina (Boghdadi and Henning, 1997; Benowitz, 1993; Prakash and Das, 1993). These effects of cocaine were also reported at the end of 1884 by Sigmund Freud, who used cocaine on himself (Das, 1993; Das and Laddu, 1993b). Months later Karl Koller announced his discovery that cocaine was an effective local anesthetic agent but also in 1884, William Halsted, a renowned surgeon at the Johns Hopkins Hospital, became the first physician to report cocaine anesthesia properties (Das, 1993; Das and Laddu, 1993b).

The first high consumption of cocaine in North America begun in the 1880 when it was first introduced (Boghdadi and Henning, 1997). By 1885 there were no laws to restrict cocaine use and it was commercially available in 15 different products such as cigarette, liquor, inhalants among others (Boghdadi and Henning, 1997). Moreover, there was a high production of coca containing wine in Europe and the United States was importing it. Even soft drinks as the famous Coca-Cola contained coca (Das, 1993; Das and Laddu, 1993b; Prakash and Das, 1993). Later in 1920’s the
consumption of cocaine was reduced because of the regulations in the distribution, implanted by the Harrison Act. Unfortunately, in the late 1970’s a second phase of high cocaine consumption began in the United States that continues today. Results from the 2012 National Survey on Drug Use and Health, reported that there were 639,000 individuals aged 12 or older who had used cocaine for the first time within the past 12 months and 1.1 million individuals are reported to show cocaine dependence or abuse. The survey also reported that 658,000 addicts received treatment for cocaine use (Substance Abuse and Mental Health Services Administration 2013).

Cocaine is a strong psychostimulant that affects monoamines concentration by blocking serotonin, DA and norepinephrine reuptake transporters. However, its main effects are related to DA neurotransmission (Woolverton and Johnson, 1992). When cocaine is in the brain, it binds to DAT which results in the blockade of DA reuptake and, thus an enhancement of DA levels within the synaptic cleft (figure 2). This blockade produces a prolonged neurotransmission of DA effects on postsynaptic neurons like the ones in the NAc that leads to pleasurable emotions and endurance of the rewarding effects (Kalivas, 2007). In this manner, cocaine produces intense euphoria, heightened energy, enhanced alertness, and increased self-confidence (Das and Laddu; 1993b; Prakash and Das, 1993). Cocaine also suppresses other brain areas resulting in the suppression of fear and panic. These account for the antianxiety effects of cocaine which may enhance its euphoric effects (Das and Laddu, 1993b; Prakash and Das, 1993). However, continuous use of cocaine can produce insomnia, anorexia, aggressiveness, depression, psychosis among others symptoms (For review, Taylor and Gold, 1990).

As mentioned before, one of the major problems of cocaine use is its long lasting effects in the brain that can drive users to relapse after long periods of abstinence. This withdrawal stage is characterized by unpleasant effects such the ones mentioned before. Cocaine relapse emerge as the
Figure 2. DA neurotransmission in the mesolimbic reward pathway. A. Normally, DA is released by a neuron into the synapse, where it can bind to DA receptors, then is recycled back into the transmitting neuron by DAT. B. If there is cocaine present, it binds to DAT blocking DA reuptake process, resulting in an enhancement of DA within the synaptic cleft and in the experience of the pleasurable effects of cocaine. (Taken and adapted from the National Institute on Drug Abuse. Cocaine: Abuse and Addiction http://www.drugabuse.gov/publications/research-reports/cocaine-abuse-addiction/how-does-cocaine-produce-its-effects)
consequence of three factors; stress, exposure to the drug or exposure to an environmental stimulus that is strongly associated with the drug experience. The more cocaine is consumed the greater will be the association of a particular environment with the effects of the drug, and eventually the greater will be the effect of environment on triggering craving and drug-seeking. When there is an encounter with that environment, or a stressor factor, it is believed that DA is released into the PFC and Amyg and mediates the reinstatement of drug-seeking (See et al., 2001; Capriles et al., 2003; Kalivas, 2007). Thus, relapse can be induced by DA released within the PFC and the allocortical brain regions triggering stimulation of glutamatergic projections between the PFC and the Amyg as well as glutamatergic outputs to the NAc and VTA (Kalivas and Volkow, 2005; Kalivas, 2007).

3. Cocaine Conditioning Paradigms with Animal Models

Associations between the effects of abused drugs and the environment during drug administration play an important role in addiction (Siegel, 1976; Stewart et al., 1984; Anagnostaras and Robinson, 1996; Hyman and Malenka, 2001; Badiani and Robinson, 2004; Wise, 2004; Everitt and Robbins, 2005; Vezina and Leyton, 2009). During cocaine use, humans can develop a strong association between cocaine rewarding effects and the environmental cues. Previous evidence shows that subsequent encounters with one of those cues can mimic the drug's locomotor-activating effects, evoke craving and precipitate relapse. It is believed that cocaine in an addicted subject shows a reduction of drug-induced DA increases but the drug will still be compulsively taken because of the process of conditioning, which is one of the neuroadaptative changes that occur after cocaine exposure (Volkow et al., 2003). This kind of conditioning involves DA signaling and synaptic changes in N-Methyl-D-aspartate (NMDA) and \( \alpha \)-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (Zweifel et al., 2009; Kauer and Malenka, 2007). Even when cocaine by itself induce an enhancement of DA signaling within the Str it is believed that with the continued use of the drug when conditioning is developed it is the
conditioned stimulus that evokes that DA release (Schultz, 2010). Moreover, glutamatergic projections from PFC into the VTA/Substantia Nigra (SN) and NAc have been shown to mediate these conditioned responses (Kalivas, 2009).

Most of the understanding about this disease and the role of the environmental cues have been obtained from animal studies. Although, today there is new technology that help us comprehend the changes that the human brain goes trough during this disease, studies with animals are still essential to understand drug addiction. Different paradigms that study the importance of environmental cues during drug exposure have been developed using animals as subjects. One example is intravenous cocaine self-administration. In this paradigm, once the animal has learned to self-administer cocaine under specific cues, they then undergo an extinction period where lever pressing has no consequence. Following extinction, animals can reinstate drug seeking just by re-exposing them to the cues previously paired with the drug (De Wit and Stewart, 1981). The cocaine self-administration studies not only have been tested with rodents, it has been extended to non-human primates, such as squirrel monkeys (Barrett-Larimore and Spealman, 1996, 1998). There are other behavioral protocols that combine environmental cues with cocaine treatment that can trigger a conditioned response. These protocols are called sensitization paradigms.

Sensitization is defined as the enhancement of a behavioral response to drugs of abuse as a consequence of repeated administration. Results from sensitization paradigms revealed that rats previously exposed to a particular drug in a drug-paired environment showed a greater locomotor response (when exposed to that environment) compared to rats previously exposed to the drug in a non-paired environment. Such environment-specific expression of locomotor sensitization has previously been reported with different drugs of abuse including morphine, amphetamine and cocaine (For review, Vezina and Marco, 2009). Another behavioral paradigm that examines conditioning effects is the conditioning place preference (CPP), in this paradigm animals are treated
Figure 3. Schematic representation of the environmental elicited cocaine conditioning develops in our laboratory
Environmental Elicited-Cocaine Conditioning

Control Subjects
- Saline IP injection

Cocaine-Paired Subjects
- Cocaine 10mg/kg IP injection
- 1:30 minutes
- Saline IP injection

Cocaine-Unpaired Subjects
- Saline IP injection
- 1:30 minutes
- Cocaine 10mg/kg IP injection

Repeat the last 3 steps for 10 consecutive days

Day 11
Rest Day

Day 12 Reexposure to environmental cues

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with drug-environment pairings, and with non-drug paired environments. During the training sessions, some days animals will receive cocaine injections and exposed to a specific environments, and in others they will receive saline injections and will be exposed to a different environment. The test day consists of a drug-free trial in which the rat is allowed free access to both the drug-paired and a non-drug paired environments (Calcagnetti et al., 1995). Usually, rats will spend more time in a context in which they passively-received the drug than in a location paired with no drug.

A similar procedure is used to test animals in cocaine-conditioned locomotion. In this protocol, animals are injected with cocaine in a drug-paired environment and the locomotor activity patterns are measured. At the same time a different group of animals is injected with saline and exposed to environmental cues. This is repeated for several days and then during the test sessions, animals are re-exposed to the cocaine paired environment and locomotors responses are measured. Results with this kind of protocol showed an enhanced response in drug-paired environment animals on conditioned locomotion (Vezina and Marco, 2009).

Based on previous experiments, an environmental elicited-cocaine conditioned locomotion paradigm was developed in our laboratory. Our conditioning paradigm is an adaptation of the previously published conditioned locomotion protocol developed by Brown and Fibiger (1992). The paradigm used in the present thesis was previously tested in our laboratory as described in Rodriguez-Borrero et al., 2006, 2010. We developed a protocol in which there are three different groups (figure 3), one of the groups was the control subjects who received saline intraperitoneal (ip) injection before placing them into the activity chambers, the cocaine-paired animals received 10mg/kg ip injection of cocaine and the cocaine-unpaired also received saline ip injection. After the injections, animals are placed into the activity chambers for one hour and thirty min. Activity chambers for control and cocaine-unpaired animals include black and white boxes in a room impregnated with nutmeg oil. For the cocaine-paired group the boxes were black and the essence in the room was orange oil. Once the time was completed, the control subjects received saline ip
injections, while the cocaine-paired subjects and the cocaine-unpaired received 10mg/kg ip injection of cocaine and then they were returned to their home cages. This was repeated for 10 consecutive days, on day 11 they rested and on day 12 they were re-exposed to the activity chambers but without any cocaine priming. It is important to clarify that cocaine paired animals were treated in a different environment than the other group but this environment by itself has nothing to do with the locomotor response on the test session (results will be presented on chapter III). This protocol aims to establish the role that environmental cues have on induced locomotor response just by the exposure of the stimulus. We consider that our paradigm is not a sensitization one because it does not include a cocaine injection during the test session on day 12, neither it is a CPP paradigm, since the animals are not exposed to two different environments to choose between them.

Several studies have been made using the different protocols described above to study proteins, gene, receptors or neurotransmitter in different brain regions (such as the NAc) involved in cocaine effects. For example, psychostimulant sensitization studies with rats have reported the enhancement of DA overflow within the NAc (Hamamura et al., 1991), and locomotion conditioning studies showed that environmental stimuli previously associated with daily cocaine administration can modulate glutamate transmission in the NAc (Bell et al., 2000). Moreover, our lab (Rodriguez et al. 2006, 2010) showed the role of glutamate ionotropic receptors in the environmental elicited cocaine conditioning expression. These results demonstrated that the cocaine-paired environment affects several neurotransmitter systems within the NAc.

4. Neuroanatomy and Neurocircuitry of the NAc

The NAc is localized in the forebrain and is the main structure of the ventral Str (Smith and Bolam, 1990), in human its dimensions are approximately 10.5 mm in length, 14.5 mm of width and 7.0 mm in height (Neto et al., 2008). In the rat, the NAc is beneath the caudate putamen and
above the olfactory tubercle (Zahm and Brog, 1992). There are two main types of cells in the NAc: medium spiny neurons and large aspiny neurons which are cholinergic interneurons (Chronister et al., 1981). The main neurotransmitter of the medium spiny neurons is GABA but there are some neuropeptides in those cells, such as neotensin, enkephalin, and substance P among others (Pickel et al., 1988; Zahm & Heimer 1988; Hussain and Totterdell, 1994; Harlan and Garcia, 1998). In the interneurons you can find GABA and acetylcholine as neurotransmitter (Hussein et al., 1996) and neotensin, calbindin, cholecystokinin and vasoactive intestinal peptide as neuropeptides (Theriault and Landis, 1982; Tagaki, 1984; Hussain and Totterdell, 1994; Delle-Done et al., 1996).

The NAc contains a complex neurocircuit, in which one of the principal innervations is the dopaminergic afferents from the VTA (Voorn et al., 1986). The dopaminergic projection from the VTA to the NAc drives the rewarding effects of drug use but it seems that the principal role is to modulate excitatory glutamatergic inputs to the NAc (Kupfermann, 1979). The NAc receives excitatory innervations from limbic structures such the ventral HPC and the basolateral Amyg and also receives glutamatergic inputs from the PFC (Groenewegen et al., 1999). In addition, it receives serotonergic afferents from the raphe nucleus, and noradrenergic afferents from the nucleus tractus solitaries and the locus coeruleus (For review Shirayama and Chaki, 2006). Once the NAc integrates these corticolimbic inputs, it projects to other basal ganglia region such the ventral pallidum (VP) (Groenewegen et al., 1999). The VP, in turn, sends feedback projections into the PFC (O’Donnell et al., 1997). This neurocircuitry between NAc, VTA, and limbic structures (HPC and Amyg) makes the NAc a center of integration of emotional, contextual and executive/motor processes (Grace, 2000; Goto and Grace, 2008). In terms of efferent projections, the NAc innervates the VP, the basal forebrain, and some regions of the globus pallidus, SN, VTA, preoptic area, hypothalamus and some caudal mesenphalic regions (For review see Basar et al., 2010; Groenewegen et al., 1993, 1996; Groenewegen and Russchen, 1984; Heimer et al., 1991). Those afferents and efferents are connections of the NAc as a whole, but the NAc is commonly divided
anatomically and functionally into two subregions: the shell and the core. Even when the NAc shares connections between its subregions, these connections are inhomogeneously distributed and some afferent projections present more relationships with the shell than the core and vice versa. Thus, projections from the hippocampal subicular and CA1 regions predominantly target the NAc shell. In addition, some PFC subregions such as, the ventral agranular insular cortex, infralimbic cortex and the ventral prelimbic cortex preferentially innervate the NAc shell. On the other hand, the dorsal agranular insular cortex and the dorsal prelimbic cortex of the PFC target the NAc core. Areas from rostral and caudal region of basal amygdaloid complex innervate the shell and those from mid rostracaudal, the NAc core. The anterior paraventricular nucleus and the posterior paraventricular nucleus show a stronger projection to the shell whereas intermediodorsal nucleus and central medial nucleus of the thalamus prefer the NAc core. Dopaminergics cells A10 from VTA predominantly innervate the NAc shell but are not restricted to only that subregion since they also connect to the NAc core. A8 project more laterally in the NAc shell whereas the A9 project in the ventral region of the NAc core (For review, Groenewegen et al., 1999).

The principal efferent of the NAc is into the VP. The shell projects to the ventromedial and ventrolateral part of the VP, while the core to the dorsal part of the VP and the VP send reciprocal innervations to the NAc (For review, Groenewegen et al., 1999). There are other efferents into dopaminergic nucleus areas. These include the ones that the shell sends to the medial and lateral regions of the VTA, the dorsal tier of the SN pars compacta and to the retrorubral area (For review see Groenewegen et al., 1999). It is also known that the NAc core innervates the SN and other regions such as the pars reticulata. Moreover the NAc shell projects preferentially to other subcortical limbic regions including some regions of the hypothalamus (For review, Groenewegen et al., 1999). In contrast, the core outputs are similar to those of the basal ganglia that produce the motor response. Outputs through VP, SN and subthalamic nucleus in turn project via motor areas (For review, Day and Carelli, 2007). Even when there is some specificity that makes possible to
5. Neurochemistry and Function of NAc in Cocaine Addiction

The NAc is a crucial brain region to understand of different behaviors including the ones associated with cocaine addiction because of its different connections with brain regions that drives motor response and, with brain areas that modulates limbic response. This brain region has been previously described as an interface, where corticolimbic information is integrated with outputs to the motor system. (Mogenson and Yim, 1980). Thus, the NAc is involved in mediating learning, reward, motivation and locomotor response. The NAc as mentioned before contains gabaergic neurons that projects to different brain regions. GABA is the most abundant inhibitory neurotransmitter in the mammalian brain and within the NAc one can find two types of GABA receptors (GABA$_A$ and GABA$_B$) (Matsumoto, 1989). GABA$_A$ receptors consist mostly of postsynaptic receptors, whereas GABA$_B$ receptors are localized presynaptically and postsynaptically (Matsumoto, 1989). These gabaergic receptors within the NAc can modulate drug abuse responses and reward processes. In fact, a study reported that GABA$_B$ receptor agonist pretreatment in rats, antagonized cocaine and morphine induced DA release in the NAc (Fadda et al., 2003). Moreover, extensive evidence suggests that there are interactions between GABA$_A$ and DA neurons (For review, Ikeda et al., 2012).

One of the principal afferents to the NAc arises from VTA dopaminergic neurons. DA is known as the pleasure neurotransmitter and plays a crucial role in modulating the rewarding effects of drugs. The NAc expresses both families of DA receptors (DA1 and DA2 receptor family). These DA receptors are present presynaptically in projections coming from limbic and cortical regions (Tarazi et al., 1998) and postsynaptically in NAc neurons (Sesack et al., 1994; Hara and Pickel, 2005). Microdialysis studies revealed that addictive drugs caused an augmentation of extracellular DA within the NAc (Imperato and Di Chiara, 1986; Imperato et al., 1986b; Di Chiara and Imperato,
Specifically, an increase of DA within the NAc shell (as compared to the core) has been observed in rats after non-contingent exposure to drugs of abuse (Pontieri et al., 1995, 1996; Tanda et al., 1997). Moreover, different studies targeting NAc DA receptors revealed the role that these receptors have in modulating the cocaine rewarding effects. For example, previous studies have shown that direct microinjection into the NAc of a selective DA1 receptor antagonist (SCH23390) in animals previously trained to self-administer, increased cocaine self-administration (Maldonado et al., 1993). These data suggest that there is a disruption of the reinforcing properties of cocaine and that DA1 receptors within the NAc are important in triggering cocaine effects. Similarly, administration of DA antagonists directly into the NAc increases the rate of psychostimulant self-administration (Maldonado et al., 1993; Phillips et al., 1994). Lesions studies have also been performed in order to understand the DA role within the NAc. For example, 6 Hydroxy DA (6-OHDA) lesion of the NAc resulted in long-lasting reduction and extinction-like responses of both cocaine and amphetamine self-administration (Roberts et al., 1977, 1980; Pettit et al., 1989). Although, DA has a huge relevance in modulating cocaine reward effects within the NAc, other neurotransmitters are also involved in this response.

As previously mentioned the NAc also receives acetylcholine innervations and contains aspiny cholinergic interneurons. It has been reported that acetylcholine enhancement within the NAc prevents addictive behavior following cocaine and morphine treatment (Hikida et al., 2003) and that acetylcholine release within the NAc increased response during the withdrawal period from drugs of abuse (Rada et al., 1991). It also has been found that cholinergic interneurons interact with dopaminergic and, glutamatergic input and with gabaergic projection neurons (Meredith et al., 1993). Moreover, cholinergic interneuron within the NAc seems to plays an important role in modulates medium spiny neurons activity and cocaine conditioning (Witten et al., 2010). Another neurotransmitter that can be found within the NAc is serotonin. It seems that serotonergic neurons within the NAc play an important role in motivation, reward and stress (McEwen et al., 1993; Nakahara et al., 1989). Application of serotonin into the NAc has been reported to stimulate DA
release (Parsons and Justice, 1993), serotonin also interacts with dopaminergic inputs (Parsons and Justice, 1993) and seems to have a neuromodulator role within the NAc shell gabaergic neurons (Van Bockstaele et al., 1996).

The NAc also receives important excitatory glutamatergics afferents from HIP, Amyg and PFC. It is postulated that glutamatergic innervations within the NAc drives and controls relapse after drug extinction (For review, Quintero, 2013), and that glutamate neurotransmission is implicated in modulating the reinforcing properties of psychostimulant reward. Glutamate is the main and most abundant excitatory neurotransmitter in the brain. Previous studies revealed that as with DA, cocaine exposure can modulate glutamate levels within the NAc (Kalivas and Duffy, 1998). Not only the drug itself, but also environmental cues associated with cocaine, increased glutamate release in the NAc (Hotsenpiller et al., 2001). Glutamate release from PFC is disrupted after chronic drug intake (For review, Quintero, 2013). Studies revealed that chronic cocaine exposure decreased glutamate levels within the NAc but a cocaine challenge pairing with environmental stimuli increase extracellular glutamate levels, and it is regulated by the pairing of environmental stimuli with drug administration (Hotsenpiller et al., 2001; Bell et al., 2000). As a consequence of the decrease of glutamate levels after chronic drug exposure the brain seems to make adjustment and there is an increase in surface expression of AMPA Receptor Subunit GluR1 (GluA1) (Conrad et al., 2008; Moussawi et al., 2009). This alteration in AMPA receptors in the NAc depended on different factors related with cocaine exposure, such as, contingency of the exposure, withdrawal duration, extinction training, or on the cues paired with cocaine (For review, Wolf and Ferrario, 2010). Different pharmacology studies that target glutamate receptors revealed that microinjection of AMPA as an agonist into the NAc induced cocaine reinstatement and inversely microinjection of an AMPA antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione blocked the reinstatement (Cornish and Kalivas, 2000). Other pharmacology studies with the ionotropitic glutamate receptor (NMDA) have shown effects on cocaine seeking behavior. Infusions of (2R)-amino-5-phosphonovaleric acid (AP5), an NMDA antagonist in any of the NAc subregions induces
reinstatement of cocaine-seeking behavior (Park et al., 2002; Famous et al., 2007). These results suggest that AMPA and NMDA receptors within the NAc may play opposing roles in the reinstatement of cocaine seeking. Other kinds of glutamate receptors (mGluRs) within the NAc shell have also been shown to modulate cocaine response (mGluRs within NAc shell is discussed further ahead).

Dopaminergic and glutamatergic projections into the NAc are indispensable to guide the rewarding effects of cocaine and also to drive relapse (as described above), but it seems that these projections do not work independently at all. Evidence indicates that there are interactions between dopaminergic and glutamatergic inputs in the NAc (Sesack and Pickel, 1990, 1992). Different studies demonstrate that glutamatergic agonists enhance DA release in the NAc (Imperato, 1990), whereas accumbal DA increase extracellular glutamate levels (Dalia, 1998). Moreover, it has been reported that NMDA receptors are co-localized on the neurons that contain DA1 receptors in the NAc shell (Hara and Pickel, 2005), and that stimulation of DA1 receptors in the NAc shell phosphorylate AMPA receptors (Carr et al., 2010).

6. Glutamate and mGluRs within the NAc in Cocaine Addiction.

It is known that approximately 80–90% of synapses in the brain are glutamatergic, and that up to 90% of neurons use glutamate as a neurotransmitter (Siegel et al., 2006). This neurotransmitter plays an important role in synaptic plasticity related to adapted behaviors (Abraham, 2008). Moreover it is well established that glutamate mediates learning and memory in other brain areas such as the HPC. As an effector of synaptic plasticity, learning and memory, glutamate and its projections to the NAc are implicated in drug addiction pathology.

Glutamate is synthesized from glucose in presynaptic cells. It can also originate from glutamine that is synthesized in glial cell. Glutamine is released into the extracellular fluid, and transported into the nerve terminals in where the enzyme glutaminase converts glutamine into
glutamate (For review, Schmidt and Pierce, 2010). Once in the nerve terminal, glutamate is incorporated into vesicles by any of the vesicular glutamate transporters (VGLUT1, VGLUT2 or VGLUT3) (For review see Schmidt and Pierce, 2010). Once glutamate is released into the synaptic cleft it can bind and exert its action through presynaptic, perisynaptic or postsynaptic ionotropic or metabotropic glutamate receptors. The ionotropic glutamate receptors which are ligand-gated receptors that mediate fast excitatory neurotransmission include AMPA, NMDA and Kainate (KA) receptors (Ozawa et al., 1998). Whereas mGluRs include eight different subtypes (mGlur1-8) that modulate pre and postsynaptic responses through G protein activation of second-messenger cascades (Ozawa et al., 1998) (figure 4). After exerting their response, glutamate signaling is terminated by EAA transporters (EAAT1-5) (Beart and O’Shea, 2007).

It is well known that cocaine influences glutamate transmission in the NAc, this influences, produces persistent changes in neuronal function which is translated into the behavioral effects of cocaine (For review, Schmidt and Pierce, 2010). Acute exposure of cocaine has little or no effect on extracellular glutamate levels within the NAc (Smith et al., 1995; Pierce et al., 1996; Miguens et al., 2008). Although there is some reported evidence of an increase in glutamate levels within the NAc (Smith et al., 1995). However, withdrawal from chronic exposure to cocaine reduces extracellular glutamate levels in the NAc (Pierce et al., 1996; Baker et al., 2002; Schmidt et al., 2005). Glutamate receptors including ionotropics and metabotropics are well distributed all over the brain. mGluRs are specifically localized presynaptically, postsynaptically and perisynaptically in neurons and glia and there are some characteristics among mGluRs (sequence homology, G-coupling and ligand selectively) that have been used to classify the mGluRs subgroups. Group I mGluRs are mostly localized postsynaptically (For review, Niswender and Conn, 2010) and they are coupled to a Gq protein which initiates Phospholipase C/Inositol 1,4,5-triphosphate/diaclyglycerol (PLC/IP3/DAG) cascade (Conn and Pinn, 1997). Activation of Group I mGluRs lead to increase cell depolarization and neuronal excitability because of ionotrophic channel modulations (Conn and Pinn, 1997).
However, group I can also be found presynaptically, perysinaptically and in glial cells (For review, Niswender and Conn, 2010). In contrast, group II and group III mGluRs are often localized presynaptically where their principal function is to inhibit neurotransmitter release (For review, Niswender and Conn, 2010) (Figure 5). mGluR group II and group III are associated to Gi that inhibit adenyl ciclase resulting in a decrease of cyclic adenosine monophosphate (cAMP) levels (Conn and Pin, 1997). These mGluRs function at excitatory (glutamatergic), inhibitory (GABAergic), and neuromodulatory (i.e. monoamines, ACh, peptides) synapses (For review see Niswender and Conn, 2010). Even when there is similarity of mGluRs within groups, there are physiological roles for mGluR subtype that are highly specific to the neuronal population and even subcellular localization (For review, Niswender and Conn, 2010). Within the NAc these receptors modulate cocaine rewarding effects and its functionality is affected by cocaine exposure.

It has been found that mGluR1 and mGluR5 are downregulated within the NAc after withdrawal from chronic cocaine exposure (Swanson et al., 2001; Ary and Szumlinski, 2007; Mitrano et al., 2008; Ghasemzadeh et al., 2009; Kalivas, 2009). In addition, group II mGluR2/3 is also downregulated after chronic cocaine exposure (Xi et al., 2002). As mentioned above, mGluR5 are Gq coupled and bind to Homer within the postsynaptic density (Szumlinski et al., 2006). Allosteric modulators of mGluR5, or deletion of mGluR5 gene reduce self-administration and
Figure 4. Glutamate receptor–mediated signaling. Glutamate released into the synaptic cleft binds to and activates ionotropic glutamate receptors (NMDA, AMPA, and KA receptors) on postsynaptic membranes. Extracellular glutamate also binds to and activates perisynaptic mGluRs located on presynaptic (mGluR2/3 autoreceptors) or postsynaptic (mGluR1/5s heteroreceptors) membranes. (Taken from Schmidt and Pierce, 2010).
drug seeking reinstatement of most abused drugs (Chiamulera et al., 2001; Olive, 2010). Mice lacking mGluR5 gene do not self administer cocaine and in another set of animals, cocaine exposure was unable to increase locomotors activity (Chiamulera et al., 2001). Moreover, mGluR5 antagonists attenuated cocaine priming- and cue-induced reinstatement of cocaine seeking (Kumaresan et al., 2009). In this experiment, rats were trained to self-administer cocaine, the lever pressing was extinguished and then animals were induced to the reinstatement of lever pressing. These results clearly demonstrated the importance of this receptor in modulating the behavioral effects of cocaine. Most of the evidence targets mGluR5 within the NAc in modulating the rewarding effects of cocaine, however there is also evidence that associate mGluR1. In sensitization experiments, rats received systemic injections of cocaine or saline, and before test session animals were pretreated with an mGlur1 antagonist (Dravolina et al., 2006). Inhibition of mGluR1 significantly reduced expression of cocaine-induced psychomotor sensitization. In experiments of cocaine-seeking behavior in rats, it was demonstrated that mGluR1 blockade within the NAc impaired cocaine seeking behavior (Xie et al., 2012). Moreover, recent data where CPP was used, postulated that mGluR1 antagonism inhibited de novo protein synthesis which may block the formation of cocaine–cue associations and thus provide a mechanism for the reduction in CPP to cocaine (Yu et al., 2013). It is clear the involvement of mGluR group I in modulates cocaine rewarding and the expression of cocaine effects. However, it is not well establish the role of mGluR group I during cocaine locomotion conditioning.

As previously mentioned glutamatergic neurotransmission within the NAc is important for the expression of psychomotor effects. Group II and group III mGluRs are well expressed within the NAc (Ohishi et al., 1993a, 1996b, 1995; Testa et al., 1994), and this family of receptors also modulates psychostimulant effects. Knockout (KO) mice for mGluR2 exhibit a higher dopaminergic activity within the NAc and an increased locomotor sensitization and CPP with cocaine exposure (Morishima et al., 2005). Similar results were obtained with pharmacological
manipulations of these receptors (Jeong-Hoon and Vezina, 2002). In a sensitization experiment, rats
were injected with amphetamine and after two weeks of the last amphetamine exposure, a group of
animals was challenged with amphetamine and another set of animals with amphetamine and a
group II agonist (Jeong-Hoon and Vezina, 2002). Results from this study showed that in animals
challenged with amphetamine in conjunction with the group II agonist, the amphetamine
sensitization was blocked (Jeong-Hoon and Vezina, 2002). Moreover, CPP was also impaired with
blockade of group II mGluRs within the NAc (Gerdjikov and Beninger, 2006). Another study with
non-human primates investigated interactions between group II mGluRs agonist and cocaine
reinstatement response (Adewale et al., 2006). After squirrel monkeys were trained to self-
administer cocaine, they extinguished the behavior, and subsequently reinstated it with a cocaine-
priming injection. Pretreatment with group II agonist reduced cocaine self-administration and
reinstatement (Adewale et al., 2006). It seems that group II mGluRs negatively modulates cocaine
effects since results showed that activation of this group with agonists can attenuate the reinforcing
and priming effects of psychostimulants. In both experiments, agonists were systemically injected,
but others studies have investigated a more specific role of this group of receptors within brain
regions. For example, in microdialysis performed in rats, local administration of group II agonist
reduces NAc shell DA levels (Greenslade and Mitchell, 2004). In contrast, local injection of group
II/III antagonist increased DA levels in the NAc (Kalivas et al., 1993; Hu et al., 1999). The same
result was obtained in slices of rat NAc where DA levels were significantly attenuated by group II
mGluR antagonists (Shigeyuki et al., 2006). Taken together these data suggest that it is possible that
group II mGluRs modulate psychostimulant rewarding effects by negatively regulating DA release
within the NAc.
Figure 5. Schematic representation of mGluRs at the synapse. In general, group I mGluRs are localized postsynaptically, group II and III receptors are mostly presynaptically. mGluRs 2, 3, 4, and 8 are generally found in extrasynaptic locations at presynaptic level, and mGluR7 is in synaptic zone. Group II and III receptors inhibit release of glutamate or GABA, in contrast group I promote release. Group I mGluRs increase intracellular calcium (Ca$^{2+}$); additionally, mGluR5 and NMDA receptors are closely linked signaling partners. Postsynaptic mGluR2/3 inhibits cAMP. mGluR3 and mGluR5 in glial cells seems to regulate synaptic activity. (Taken from, Niswender and Conn, 2010).
Group III mGluRs studies in addiction are scarce. As mentioned before, these receptors inhibit adenyl cyclase and are mainly found presynaptically. They are expressed within the Str regions, and as with mGluR group II, microdialysis experiments showed that striatal perfusion of an mGluR group III agonist reduced DA levels within the NAc. In contrast, perfusion of an antagonist enhanced DA levels (Hu et al., 1999; Mao et al., 2000a, b). In terms of relation with psychostimulants, it has been found that mGluR group III antagonist attenuated motor response produced by psychostimulants exposure (Mao et al., 2000a). In this experiment, hyperlocomotion response caused by acute cocaine or amphetamine injections was blocked by microinjection of group III mGluR agonist within the Str (Mao et al., 2000a). In addition, a microdialysis study showed that perfusion of amphetamine (within the Str) enhanced extracellular DA levels, but when amphetamine was co-perfused with group III agonist, the amount of extracellular DA was reduced (Mao et al., 2000b). As expected, these data suggested that group III mGluR within Str had a similar role to the group II mGluR.

As explained in the past section, mGluRs within the NAc and the Str modulated psychostimulants response. However, as is already known, this family of receptors activates an intracellular cascade of events and also interacts with different proteins that for instance modulate the mGluRs response. mGluR group I interacts with Homer a scaffolding protein that seems to be involved in modulating cocaine response and also its protein expression is affected with cocaine exposure.

7. Homer, Its Role in Cocaine Addiction and mGluR5 Interaction

Homer is a family of proteins that are encoded by three genes (Homer1-3) (Brakeman et al., 1997; Kato et al., 1998; Xiao et al., 1998). These three different genes give rise to constitutively expressed, long isoforms (Homer1b/c/d, Homer2a/b, and Homer3) and, immediate early gene (short) isoforms (Homer1a and aina-3) (For review, De Bartolomeis and Iasevoli, 2003). The two isoforms contain an Ena/VASP1 homology (EVH1) domain that interacts with a long proline-rich
motif located on Group 1 mGluRs, inositol-1,4,5- triphosphate (IP3) and ryanodine receptors, transient receptor potential (TRP) cation channels and Shank (Brakeman et al., 1997; Kato et al., 1998; Xiao et al., 1998; Tu et al., 1998, 1999; Hwang et al., 2003; Kammermeier et al., 2000; Yuan et al., 2003). Homer long isoforms contain at the C-terminus coil-coil (CC) and leucine zipper-motifs domain that interact with glutamate receptors and enables their clustering with proteins involved in their intracellular and, cell membrane localization (Naisbitt et al., 1999; Abe et al., 2003; Rong et al., 2003; Shiraishi et al., 2003 a, b). On the other hand, the short isoform of homer1 does not contain a CC-domain and the ability to multimerize (Xiao et al., 1998; Bottai et al., 2002). The lack of CC-domain in Homer1a prevents this protein to form dimers. However, it competes with Homer long isoforms and disrupts the scaffold provided by the long Homer proteins. Thus, Homer1a acts as a dominant negative binding protein (For review, Ulrich, 2002; Fagni et al., 2002) (figure 6)

Previous studies related this family of proteins to cocaine exposure, and showed that Homer isoforms are differentially regulated within brain regions that modulate neuroplasticity in addiction (For review, Szumlinski et al., 2008). For example, acute cocaine transiently elevated short Homer isoforms, while a reduction in long Homer isoforms was observed following withdrawal from repeated cocaine administration (For review, Szumlinski et al., 2008). More specifically, it has been shown that (in mice and rats), withdrawal from repeated cocaine administration downregulated Homer1b/c and Homer2a/b within the shell, but not the NAc core, and the reduced Homer levels were accompanied by decreases in mGluR1a, NMDA receptor subunit 2a and 2b expression (Ary Szumlinski, 2007). Another study demonstrated that there was a correlation of decreased amount of mGluR5 and Homer1b/c within the NAc shell after extinction of cocaine seeking behavior (Ghasemzadeh et al., 2009).

In addition to its expression, Homer’s role during cocaine treatment has been investigated. For example, mutant mice for Homer1 or Homer2 presented a response to cocaine comparable with
animals that have been in withdrawal from repeated cocaine exposure (Szumlinski et al., 2004a). In this experiment, Homer1 or Homer2 KO mice showed higher cocaine CPP, higher response to acute cocaine exposure and a better acquisition of cocaine self-administration when compared with wild type (Szumlinski et al., 2004a). Moreover, basal glutamate levels within the NAc was approximately 50% less than wild type, as it happens with cocaine withdrawal mice (where there is less glutamate) (Szumlinski, et al. 2004a). In addition, an acute injection of cocaine in both KO mice produces an increase in glutamate levels within the NAc, similar to what has been shown when cocaine withdrawal animals receive a cocaine challenge. Interestingly, this withdrawal cocaine phenotype in these Homer2 KO mice was reversed with Homer2b microinjection directly into the NAc. This data clearly demonstrate that Homer1 and Homer2 modulated both cocaine sensitivity response and the neurochemical effects of acute cocaine. Another study aimed to establish if downregulation of the long Homer isoforms (following withdrawal from repeated cocaine administration) was necessary for enduring alterations in cocaine-induced changes within the brain. In this experiment, they overexpressed short and long isoforms of Homer within the NAc of rats and found that overexpression of the long homer isoform abolished cocaine-induced sensitization of locomotor hyperactivity and prevented the development of glutamate abnormalities within the NAc (Szumlinski et al., 2006a).

As previously mentioned, Homer proteins can bind to group I mGluR receptors. Cell culture studies revealed that Homer may regulate the expression and function of groupI mGluRs at different steps such as surface expression, clustering, physical linkage to other synaptic and subsynaptic complexes among others (For review, Szumlinski, et al. 2006b; Ulrich, 2002). Different cell cultures studies have shown mechanism where Homer isoforms interact and modulate group I mGluR into the postsynaptic density. For example, when cerebellar granule cells were co-transfected with Homer1b/c and mGluR5 there was postsynaptic accumulation of Homer-mGlu5 receptor complex (For review, Fagni et al., 2002). Another study showed that Homer1a targeted
mGluR5 toward dendrites and the axons (For review, Fagni et al., 2002). Further studies postulated that Homer1b/c-mGluR5 are indeed retained in the cytoplasm and depolarization-induced Homer1a expression triggered the translocation of the mGluR5a to the plasma membrane, at the same post-synaptic sites (For review, Fagni et al., 2004). Not much is known of what is the specific role of Homer1 in mGlur5 cellular localization. A model that attempted to explain what is happening with mGluR5 trafficking revealed that, resting neurons constitutively expressed Homer1b and Homer1c proteins. In addition, Homer 1b/c proteins created an intracellular pool of mGluR5 at post-synaptic sites. Once intense neuronal activity occurs, the induced Homer1a synthesis would promote the translocation of the intracellular mGluR5 to the post-synaptic membrane (Ango et al., 2002; Fagni et al., 2002).

Homer long isoforms contain a CC-domain that gives them the ability to dimerize and connect group I mGluRs with IP3 receptors (For review, Ulrich, 2002). This means that this long isoforms not only modulates mGluRs membrane expression but also mGluRs signal transduction. It has been shown that at three weeks after discontinuing one week of daily cocaine injections, the capacity of mGluR group I agonist DHPG (within the NAc) to induce glutamate release and induce an mGluR1-dependent increase in locomotor activity was reduced. This reduction correlated with a decrease in the Homer1b/c protein (Swanson et al., 2001). In striatal neurons, the Homer 1b/c isoform protein is the one that linked mGluR5 to ERK1/2, disrupting the mGluR5-Homer1b/c association (or the PI/Ca2+ cascade) disrupts cAMP response element-binding protein (CREB)/Elk-1 and c-Fos stimulation (Mao, 2008). In fact, this study demonstrated that Homer 1b/c modulated
**Figure 6. Homer short and long isoforms** (a) Homer CC multimers provide a coupling between mGluRs, IP3Rs and the PSD, thus connecting mGluRs to the NMDA receptor signalling complex. (b) Homer 1a interferes with multimerization.

*(Taken from Szumlinski et al., 2004a)*
mGluR5 intracellular cascade. Moreover, a more direct relation of mGluR5-Homer interaction in modulating cocaine response was demonstrated by microinjecting into the NAc core a membrane permeable peptide that disrupts this interaction. Inhibiting mGluR5-Homer interaction within the NAc core, resulted in the inhibition of cocaine seeking behavior (Wang et al., 2013). However, mGluR5 expression did not change, which revealed that the peptide did not alter the surface trafficking of mGluR5. It is suggested that the behavioral result from the disruption of the intracellular signaling was mediated by mGluR5-Homer interactions.

8. ERK1/2 signaling cascade and its role in cocaine addiction

Cocaine addiction involves a serial of neurochemical adaptations within the reward pathway which make this disease a difficult one to be eradicated. These adaptations involve modifications in neuronal mechanisms that underlie learning and memory processes (Hyman et al., 2006). Although many of the drugs interact with proteins at the extracellular levels, the effects that they elicit can be a consequence of the intracellular signaling cascade actions. The intracellular signaling cascades include several proteins such as enzymes, (i.e. kinases), that lead to the activation of transcription factors, changes in gene expression, and modifications in synaptic plasticity. One of those intracellular cascade proteins that regulate learning and memory processes is ERK, a member of the mitogen-activated protein kinase (MAPK) intracellular signaling pathway (Adams and Sweatt, 2002; Berman and Dudai 2004).

The ERK kinase family (ERK1–8) is characterized by a threonine (Thr)/ tyrosine (Tyr) motif that need to be phosphorylated for its activation. The two main isoforms of ERK are 44 kDa (ERK1) and 42 kDa (ERK2). ERK1/2 are involved in cell growth and differentiation, and also in neuronal plasticity (Thomas and Huganir, 2004; Sweatt, 2004). Upon ligand activation of Tyr kinases or G protein coupled receptors, MAPK intracellular cascade is activated. First, small G proteins of the Ras family activate MAPK/ERK-kinase-kinases (MEKKs) of the Raf family (Kolch, 2005). This action activates MEK1/2, which leads to the phosphorylation of ERK1/2. ERK1/2
activation subsequently activates different transcription factors such as CREB and Elk-1 (Sweatt, 2001).

Previous studies have demonstrated that ERK1/2 were expressed within mesocorticolimbic regions of the brain after acute or repeated treatment with psychostimulant (Berhow et al., 1996; Valjent et al., 2000, 2004, 2005; Choe et al., 2002; Zhang et al., 2004; Rajadhyaksha, 2004). Moreover, ERK1/2 signaling has been found to modulate several cognitive processes and the neurobiological effects of psychostimulant drugs (Orban et al., 1999; Fasano and Brambilla, 2002). KO studies for ERK1 showed that deletion of ERK1 isoform, leads to an increase in ERK2 stimulus-dependent signaling, which results in the facilitation of the development of cocaine-induced psychomotor sensitization and the acquisition of a cocaine conditioned place preference (Ferguson et al., 2006). Pharmacological studies also demonstrated that ERK1/2 manipulations modulated cocaine response. For example, Pierce et al. 1999 reported that blockade of MEK (which phosphorilates ERK1/2) within rat VTA before development of sensitization, attenuated expression of cocaine-induced locomotion sensitization (Pierce et al., 1999). In another study, similar results were obtained when blockade of MEK during development phase inhibited the expression of the conditioned locomotor responses to a cocaine associated context (Valjent et al., 2006). Not only inhibition of ERK activation within VTA disrupted cocaine response, it also has been seen that blockade of ERK phosphorylation within the NAc inhibits the expression of cocaine-induced behavioral sensitization in rats (Seungwoo et al., 2011). Moreover, pharmacological and biochemical studies showed that systemic or intraacumbens injections of MEK inhibitors blocked cocaine-induced activation of the transcription factor Elk1, CREB, Fos expression, and the activity of the intermediary kinase Mitogen- and stress-activated protein kinase 1 (MSK1) within the Str or the NAc (For review, Lu et al., 2006). Taken together, these results could implicate ERK1/2 intracellular cascade in the modulation of the neurochemical adaptations elicited by cocaine addiction.
Within different regions of the brain such as the Str, the mGluRs modulate ERK1/2 activation. For example, microinjection into the rat Str of the group I selective agonist, significantly increased pCREB, pElk and pERK levels (Choe and Wang, 2001). This increase was blocked by intrastriatal infusion of the group I selective antagonist (Choe and Wang, 2001). This induction of CREB, Elk and ERK phosphorylation through group I mgluRs seems to be mediated by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) since co-administration (within the Str) of a CaMKII inhibitor and group I mGluR agonist, (DHPG) attenuated these phosphorylations (Choe and Wang, 2001). In addition, it seems that mGluR1/5 mediated ERK1/2 activation can be reduced if Homer1b/c-mGluR5 interactions is disrupted (Mao et al., 2005). A functional link between group I mGluRs activation and ERK phosphorylation was reported by Gallagher et.al. (2004). On this study researchers demonstrated that induction of mGluR-Long Term Depression (LTD) in the HIP was dramatically reduced with the inhibition of ERK upstream kinase (Gallagher et al., 2004). Moreover, recent data indicated that Homer1c–mGluR5 interactions are necessary for the mGluR-dependent Long Term Pontentiation (LTP), and that this LTP involved Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and ERK activation (O’Riordan et al., 2014).

Since mGluR5 modulates synaptic plasticity processes and cocaine rewarding response, it is suggested that these receptors within the NAc are modulating the learning and memory processes involved in cocaine addiction through its interactions with the Homer1 proteins and the ERK1/2 signaling cascade activation.
Chapter II

mGluRs within the NAc Shell and Expression of Environmental-Elicited Cocaine Conditioning.
Abstract

The NAc plays a major role in the activating and reinforcing properties of drugs of abuse. EAA receptors within the NAc are involved in regulating the neural mechanisms underlying learning and memory processes necessary for associating environmental stimuli with drug use. The mGluRs within the NAc have been implicated in modulating psychostimulant reward. We hypothesized that blockade of mGluRs subtypes within the NAc will impair associative learning responsible for the cocaine conditioning state. Rats were implanted with cannulae within the NAc shell, and separate groups were exposed to a multimodal environment within activity chambers that signaled cocaine (paired) or saline (controls, unpaired). Prior to placing the animals in the chambers, rats received systemic injections of saline or cocaine for 10 consecutive sessions. On the test session (Day 12) separate groups of animals were infused within NAc shell with either mGluR1 antagonist 7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), mGluR5 antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), group II receptor antagonist (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), group III receptor antagonist (RS)-α-Cyclopropyl-4-phosphonophenylglycine (CPPG) or vehicle. The behavioral parameter, total move distance (TMT) and ambulatory distance (AD) was examined and used for statistical analyses. Blockade of mGluR1 subtype and blockade of group III within the NAc shell do not provoke any changes in conditioned locomotion in the cocaine-paired groups neither in the control and cocaine-unpaired when compared to their homologue that received vehicle on the test session. Blockade of group II resulted in an enhancement of movement in all the groups when compared with their vehicle homologues. In contrast, blockade of mGluR5 subtype decreased conditioned locomotion only in the cocaine-paired groups. These results suggested that the mGluRs subtypes within the NAc shell play different roles in the expression of a conditioned response elicited by cocaine use.

Introduction
Drug dependence is a psychological and physiological disease that have been under study for decades and still today there is not a cure for this disease. The continuous use of some drugs, such cocaine can cause addiction. Cocaine is a psychostimulant that affects reward neurocircuitry eliciting drug dependence and drug relapse even after long periods of abstinence.

Associations between the effects of abused drugs and the environment during drug administration plays an important role in addiction (Vezina and Leyton, 2004). It has been proved that environmental cues previously associated with drug use can trigger relapse. For example, animals can be trained to self-administer cocaine and after extinction of the behavior, animals can reinstate into drug seeking by re-exposure to drug associate cues (De Wit and Stewart, 1981). Moreover, rats previously exposed to the drug in a drug-paired environment showed a greater locomotor response compared to rats previously exposed to the drug in a non-paired environment, even though the non-paired environment rats received the same pharmacological treatment as drug-paired environment rats. Such environment-specific expression of locomotor sensitization has been reported with different drugs including morphine, amphetamine and cocaine (Vezina and Leyton, 2009). A similar procedure is used to test animals for conditioned locomotion. This procedure allows for the testing of conditioned responding when rats are pre-treated with saline before the test session. Results showed an enhanced response in drug-paired environment animals on conditioned locomotion (Vezina and Leyton, 2009).

In the brain, a group of different structures collectively called the DA mesocorticolimbic reward pathway mediate the rewarding, reinforcing and locomotion effects of drugs of abuse. In addition, this neuronal pathway regulates the emotional incentive given to the drugs, the memory processes associated with drug consumption and environment paired with drugs use (Kobb et al., 1998; White and Kalivas, 1998). The NAc which is an essential structure of this reward pathway is also the site within the corpus Str most implicated in locomotor activity driven by DA and glutamate transmission (Vezina and Kim, 1999). Moreover, previous studies revealed that
environmental cues associated with cocaine increased glutamate release in the NAc (Hotsenpiller et al., 2001). The NAc is divided into two subregions; core and shell that collectively modulate psychomotor functions and emotional processes (Brog, 1993; Zaham and Brog, 1992; Zaham and Heimer, 1993; Zaham, 1999). The different connections that emerge from each one of the subregions provide them with a specific function. NAc core is more related with locomotion while the NAc shell has been involved in regulating emotional processes related to reward (Brog, 1993; Zaham and Brog, 1992; Zaham and Heimer, 1993; Zaham, 1999). Which processes are sub-served by each region depend on their glutamatergic afferents from limbic-cortical regions (Di Ciano and Everitt, 2001).

Studies have shown that glutamate levels in the NAc are modulated by cocaine exposure. For example, acute administration of cocaine induced enhanced extracellular glutamate levels in VTA and NAc (Kalivas and Duffy, 1998). Moreover, reduced amounts of glutamate levels within the NAc were detected after chronic cocaine treatment, but a subsequent acute injection elevated extracellular glutamate levels, and is regulated by the pairing of environmental stimuli with drug administration (Bell et al., 2000; Hotsenpiller et al., 2001). The glutamate that is released within the NAc acts on both ionotropics (i.e. AMPA, NMDA) and mGluRs. Ionotropic glutamate receptors expression is modulated by cocaine exposure. Ghasemzadeh and colleagues, showed that acute cocaine exposure reduces mRNA levels of ionotropic glutamate receptors subtype 3 and 4(GluR3, GluR4) and NMDA receptor (NMDAR) 1, while chronic exposure only reduced mRNA levels of GluR3 in the NAc (Ghasemzadeh et al., 1999). Moreover, ionotrophic glutamate receptors within the NAc were also implicated in mediating motor learning and reinforcing effects of cocaine (Rodríguez-Borrero et al., 2006).

In addition to glutamate ionotrophic receptors function within the NAc, it has been showed that there is a functional interaction between mGluRs and DA terminals within the NAc (Vezina and Kim, 1999). Eight mGluRs subtypes divided in three groups (Conn and Pinn 1997) have been
characterized and subtypes from all groups are found within the NAc subregions (Kenny and Markou, 2004; Mitrano et al., 2008). Group I includes mGluR1 and mGluR5 which are coupled to a Gq protein and activate PLC. (Kenny et al., 2004). The mGluR2 and mGluR3 are classified in Group II. Group III is composed of mGluR 4, 6, 7 and 8. Both of these groups are associated with a Gi/q protein and regulate adenylate cyclase in a negative manner (Conn and Pinn, 1997).

Related with Group I studies Dravolina and colleagues (2006) reported that applying an mGluR1 antagonist reduces sensitization to chronic cocaine administration (Dravolina et al., 2006). Previously, Mitrano and colleagues (2008), showed changes in the subcellular and subsynaptic localization of mGluR Group I after chronic and acute cocaine administration (Mitrano and Smith, 2008). Moreover, knockout mice for mGluR5 or animals treated with an mGlur5 antagonist did not self-administer cocaine (Chiamulera et al., 2001; Kenny et al., 2001; Paterson and Markou, 2005). The Group II mGluRs are also involved in cocaine addiction. Group II mGluRs are located at glutamatergic terminals within the NAc and function as autoreceptors that decrease glutamate release (Zheng-Xiong et al., 2002). Knockout mice for the mGluR2 subtype showed enhanced cocaine sensitization and conditioned place preference (Morishima et al., 2005). Moreover, group II agonist decreased cocaine-seeking behavior relapsed induced by exposure to cocaine-associated stimuli (Weiss and Baptista, 2003). Stimulation of Group II mGluRs in squirrel monkeys reduced cocaine self-administration and cocaine-induced reinstatement of drug seeking behavior (Spealman et al., 2006). Less information is available for the role of mGluRs Group III in cocaine addiction. Neugebauer and colleagues (2000), reported that mGluR group II and III were effective inhibiting synaptic transmission under chronic cocaine treatment (Neugebauer et al., 2000). Furthermore, treatment with L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4), an agonist of group III blocked hyperlocomotion activity induced by acute cocaine exposure, and this blocking effect was reversed by pretreatment with (RS)-α-Methyl-4-phosphonophenylglycine (MPPG), an antagonist of Group III receptors (Mao and Wang, 2000).
Different studies have demonstrated higher levels of DA in the NAc shell than in the NAc core with drugs of abuse exposure, including psychostimulants (Zocchi et al., 2003). In self-administration paradigm it was show that rats will self-administer DA uptake inhibitor into the NAc shell but not the NAc core, suggesting that the NAc shell contributes more than the NAc core to the reward triggered by DA neurotransmission (Ikemoto, 2002). Cue induced reinstatement studies suggested that specific regions of the Str are implicated in the control of responding by drug-paired conditioned (Beninger et al., 1980; Shahan and Burke, 1980). Since the NAc shell is more related to the limbic brain structures, we hypothesized that this sub-region is involved in environmental cues-elicited cocaine conditioning.

Glutamate and its receptors within the NAc shell play different roles during cocaine treatment. Because of mGluRs innervations with limbic regions, they may be involved in cocaine conditioned behavioral response and may play a role in the learning and expression of that response. With this study, we aim to establish the role of mGluRs within NAc shell during environment-elicited cocaine conditioning expression. We hypothesized that blockade of mGluRs in the NAc shell will disrupt the conditioned locomotion induced by environmental cues. Our findings suggest a role of mGluRs receptors in the expression of cocaine conditioning.

Materials & Methods

Subjects

Male Sprague Dawley Rats (250-275 g) were used in all experiments. These animals were purchased from Charles Rivers Laboratories (Wilmington, MA). Rats were housed in pairs at the Animal Facility at the University of Puerto Rico, Rio Piedras. Animals were kept at controlled humidity and temperature, on a 12-hour light/dark cycle with food and water available at all times. All procedures were conducted according to the National Institutes of Health Guide for the use of
laboratory animals and our animal care institution (IACUC). A total of 118 animals were used for results and statistical analyses.

**Cannulae implantation surgery**

Surgery was performed as previously described by Rodríguez-Borrero E. et al. 2006. Briefly, all animals were food deprived the day before surgery. On the day of surgery rats were anesthetized with sodium pentobarbital (0.25mg/Kg) (Sigma-Aldrich, MO) and atropine sulfate was administered (0.54mg/Kg) (Phoenix Pharmaceuticals). The 10mm guide cannulae (23 gauges, Small Parts, FL, USA) were implanted using a stereotaxic procedure within the NAc shell (A-P +3.5, M-L ±1.0, D-V -5.3) and rats were placed with the nose bar 5.0 mm above interaural zero. Stainless steel screws (Plastic One, VA, USA) and dental cement (Caribbean Dental Products Corporation, PR) were used to secure the cannulae, and wire stylets were inserted into them. Rats were allowed to recover 5-7 days before starting behavioral experiments.

**Microinfusions**

For intracerebral microinfusions, we used 12.5 mm injector cannulae (30 gauge, Small Parts, FL, USA). The injector was inserted into the bilateral cannulae in the rat brain and using a microdrive pump (Harvard Apparatus) an antagonist (Tocris Bioscience, MO) or vehicle 0.5µl/side was administered through polyethylene tubing (Downing, Fisher Scientific Cayey, P.R.). Each rat received only one concentration of antagonists or vehicle and total infusion time was 1 min and 33 seconds followed by a 1 min diffusion period. During that period, the rat was hand-restrained by the investigator. After the microinfusion procedure, injectors were removed and 10 min later the testing session was conducted.

**Drugs**
Animals were injected ip with either 0.9% sodium chloride solution (Baxter Health Care Corporation, IL) (saline) or 10mg/kg of cocaine-hydrochloride (Sigma-Aldrich, MO) diluted in saline.

mGluRs antagonists were purchased from Tocris Bioscience, MO. We used CPCCOEt, at 10 nmol/5µl/side diluted at 50% Dimethyl sulfoxide (DMSO) + 50% Saline (vehicle) (Swanson and Kalivas, 2000) mGluR5 blockade was performed with 25nmol/5µl/side of MPEP diluted with 50% DMSO + 50% Saline (vehicle). For mGluR Group II LY341495 was used diluted at 1.0eq Sodium hydroxide (NaOH) (vehicle for Group II and III) and 100 µM/5µl/side of CPPG, (Shave et al., 2001; Iacovelli et al., 2002).

**Conditioning methods**

*Activity chamber apparatus:*

TruScan Photobeam Scanning System activity boxes (Coulbourn Instruments, PA USA) were used for the present experiments. The boxes are 41 centimeter (cm) × 41cm × 41 cm on the inside and 53 cm × 53 cm in the base plate. There are 16 photobeams in each box to track animal movement. The boxes are located with visual and olfactory environmental cues in a room different from the room of their home cages. The chambers used for the cocaine-paired group were surrounded by a 61 cm × 61 cm × 61 cm black plexiglass box (visual cue). In addition, California orange oil (Sigma-Aldrich, MO, USA) was released in the room to provide an olfactory cue to the chamber. For the control and cocaine-unpaired groups, the chambers were localized in a different room, surrounded by a 61 x 61 x 61 cm black and white (checkers) plexiglass box and the chamber exposed to a Nutmeg East Indian oil scent (Sigma-Aldrich, MO, USA), an (olfactory cue).

Animals were assigned to one of three different conditioning groups: cocaine-paired, control, and cocaine-unpaired.
Training session

The training consisted of a conditioning protocol of 10 consecutive days, one session per day for 90 min. Rats were trained during the light phase at approximately the same time on all days. Just before each training session started, rats assigned to the cocaine-paired group were injected i.p. with 10mg/kg of cocaine-hydrochloride. At the end of each training session, animals received an i.p. injection of saline and were returned to their home cages. In contrast, control groups received i.p. saline injections before and after each training session. The cocaine-unpaired group received i.p. injections of saline before placing them in activity chambers and 10mg/kg of cocaine at the end of the training sessions, after which the animals were returned to their home cages. Animals completed their training sessions in the same activity chamber throughout all the experiments. Two behavioral parameters were measured in all the sessions; (a) AD is measured as distance traveled on the floor plane minus stereotypic movements and (b) TMT which is the sum of the total elapsed time of all movements in the floor plane.

Histological Analysis

Frozen brains were cut in 40µm sections using a Leica cryocut cryostat. All sections were mounted in gelatin-coated slides, defatted, and stained (cresyl violet). The location of the cannulae was verified by microscope inspection. If the cannulae were outside of the NAc shell, the animals were excluded from the data analysis.

Statistical Analysis

The behavioral parameter used to analyze our data was TMT and AD. For the test session (Day 12 (D12)), expression session), data was analyzed using a Two-way analysis of variance (ANOVA) to determine whether drug conditioning depended on the antagonist used and whether there is a possible interaction of antagonist microinjection with the conditioning. Post hoc multiple comparison analyses were performed using Tukey test when significance was obtained.
Results

Histological analysis

Placement of the cannulae within the NAc shell was verified using cresyl violet staining. Figure 1 shows a schematic representation of rat brains sections with the approximate localization of the cannulae placement. Animals with cannulae outside of the area of study were excluded from analysis. Most of the cannulae where positioned between + 1.60 and 1.00 from bregma. A total of 118 rats were used for statistical analysis.

Effects of mGluRs Group I blockade within NAc shell

Animals were treated during 10 days with cocaine or saline and paired with a specific environment. On D12 the test session was performed where animals were exposed only to the environment previously paired with cocaine or saline. In order to determine mGluRs group I role during expression of the conditioning separate groups were microinjected within the NAc shell with either mGluR1 or mGluR5 antagonists. Results for AD and TMT during the first hour of the testing session of animals treated within NAc shell with CPCCOEt, MPEP or its vehicle are shown in
Figure 1. Photomicrograph of representatives animals and schematic representations of the cannulae placement within the NAc shell. Circles represent the approximate bilateral cannulae location within the NAc shell. The left side included all the animals that were microinjected with CPCCOEt, MPEP or vehicle. The right side include all animals micoroinjected with LY341495, CPPG or vehicle. A total of 118 animals were used. Injector sites may appear fewer than the reported number of rats because of the overlap of placements.
Schematic Representation of the cannulae placements within the NAc shell
When we used CPCCOEt for mGluR1 blockade, as expected cocaine-paired animals showed a higher locomotor activity when compared with control and cocaine-unpaired animals. Statistical analysis for TMT and AD showed a significant conditioning effect \((F [2, 37] = 10.112 \ p<0.01), (F [2, 37] = 17.922 \ p<0.01)\) (figure 2a, 2b) No statistically significant differences were found between conditioning and CPCCOEt animals. A post-hoc analysis demonstrated that comparisons for the conditioning factor for TMT in vehicle-treated animals, showed that the cocaine-paired group \((n=7)\) had a greater TMT over controls \((n=6, p<0.001)\) and cocaine-unpaired animals \((n=7, p<0.05)\). This result indicated that the environment-elicited cocaine conditioning was present. For AD, there was a difference between cocaine-paired and control animals \((p<0.05)\). Results showed no statistical difference between cocaine-paired animals and cocaine-unpaired animals. No significant differences were obtained in the results for the CPCCOEt treatment in any of the groups.

In the mGluR5 experiments results showed a statistically significant difference for conditioning factor \((F [2, 39] = 9.001 \ p<0.001)\). Moreover, there was statistically significant interaction between conditioning and MPEP treatment \((F [2, 39] = 5.011 \ p<0.05)\). In the AD parameter, similar results were obtained with a significant difference for conditioning \((F [2, 39] = 9.962 \ p<0.001)\) and a significant interaction between conditioning and MPEP treatment \((F [2, 39] = 8.172 \ p<0.01)\). These findings suggested that the expression of the conditioning is affected with MPEP treatment. A post hoc analysis demonstrated that when comparing conditioning within the vehicle group for TMT there was no significant difference for cocaine-paired \((n=7)\) animals when compared with control \((n=6, p<.001)\) and cocaine-unpaired animals \((n=7, p<0.05)\). For AD, cocaine-paired animals moved more than controls \((p<.001)\) and cocaine-unpaired animals even when there was no significant difference between these last two groups (Fig. 2c, 2d). Blockade of
Figure 2. Effects of mGluR1, and mGluR5 blockade within the NAc shell on drug expression session. The parameter used to measure response was the TMT and AD in one hour (measurements are in seconds. 2a. Cocaine-paired vehicle subjects presented TMT than control vehicle subjects (***p<.001) and cocaine-unpaired vehicle subjects (*p<0.05). 2b. Animals treated with cocaine and vehicle showed a a higher locomotor activity than control vehicle (*p<.05) and cocaine-unpaired. 2c. Cocaine paired vehicle subjects presented a higher TMT than control (*** p<0.001) and cocaine-unpaired vehicle subjects (*p<0.05). Blockade of mGluR5 with 25nmol of MPEP significantly decreased the locomotor activity of the cocaine-paired animals (cocaine-paired vehicle versus (vs.) cocaine-paired MPEP treated animals ($§$ p< 0.01) no difference was obtained between control subjects neither between unpaired subjects. 2d. AD showed that cocaine-paired animals presented a higher response (cocaine-paired vehicle vs. control vehicle (***p<0.01). Blockade of mGluR5 with 25nmol of MPEP significantly decreased the locomotor activity of the cocaine-paired animals (cocaine-paired vehicle vs. cocaine-paired MPEP treated animals (§§ p< 0.01) but not difference was obtained between control subjects neither between unpaired subjects.
Fig. 2

A. mGluR1 blockade within the NAc shell during expression session

B. mGluR1 blockade within the NAc shell during expression session

C.
mGluR5 subtype with MPEP decreased the locomotor activity in the cocaine-paired group \((n=7)\) when compared with the cocaine vehicle treatment group \((n=7)\) \((p<0.01\) for TMT and \(p<0.01\) for AD). In addition, there was not a significant difference in results for drug treatment between controls and cocaine-unpaired subjects.

**Effects of mGluRs Group II blockade within NAc shell**

In order to determine the role that Group II mGluRs have during the expression of environmental-elicited cocaine conditioning, we microinject LY341495, a group II mGluR antagonists within the NAc shell. Results for TMT and AD during the first hour of the testing session are shown in figure 3. A Two Way ANOVA analyses showed that for TMT a statistically significant difference was recorded for conditioning \((F[2, 37] = 14.119 \ p<0.001)\) and for treatment \((F[1, 37] = 101.897 \ p<0.001)\) A statistically significant interaction between conditioning and treatment was also found \((p<0.05)\). For the AD, results demonstrated a significant difference for conditioning factor \((F[2, 37] = 7.792 \ p<0.01)\), and treatment \((F[2, 37] = 35.851 \ p<0.001)\). A post hoc analysis showed that in the conditioning within vehicles for TMT there was a higher response in cocaine-paired \((n=6)\) animals when compared with control \((n=6, \ p<0.05)\) and cocaine-unpaired animals \((n=6, \ p<0.05)\). For the AD, there was a higher response in the cocaine-paired animals, but significant difference was not reached. Group II blockade with LY341495 enhanced locomotor activity in all group tested when compared with their respective vehicle treated group (Paired \(n=6, \ p<0.001\); Control \(n=6, \ p<0.001\); and Unpaired \(n=8, \ p<0.001\)).

**Effects of mGluRs Group III blockade within the NAc shell**

Conditioned locomotion response following the blockade of mGluR group III within the NAc shell is shown in figure 4. Statistical analysis for TMT demonstrated that there is significant difference for the conditioning factor \((F[2, 38] = 4.581 \ p<0.01)\) the same for AD \((F[2, 38] = 6.269 \ p<0.01)\). Once again for TMT a post hoc revealed that the cocaine-paired subjects \((n=6)\) showed a
higher response when compared with vehicle subjects \((n=6, \ p<0.01)\), and cocaine- unpaired vehicle treated animals \((n=6, \ p<0.05)\). For AD, there was a higher response for cocaine-paired animals but without statistical significance. Finally group III blockade with CPPG did not produce any changes on locomotor activity in any of the groups (control \(n=7\), cocaine-paired \(n=7\), cocaine-unpaired \(n=7\)).

**Discussion**

We demonstrated that continuous pairing of cocaine with visual and olfactory cues can elicit conditioned locomotion. Moreover, we showed that the expression of environmental elicited cocaine conditioning can be disrupted with mGluRs manipulations within the NAc shell. On the test session we microinjected mGluRs antagonists within the NAc shell, different effects were found depending in which mGluR subtype was blocked. MPEP, antagonists of mGluR5 subtype reduced conditioned locomotor activity induced by the environmental cues previously paired with cocaine injections. This reduction was not found in the control and unpaired subjects, implicating this receptor modulating the expression of cocaine conditioning effects. In contrast, microinjection of LY341495 within the NAc shell enhanced the locomotion response in all three experimental groups. This implies that this antagonist at the concentration used potentiated the locomotor response independently of what environmental conditioning the animals were receiving. CPCCOEt, an mGluR1 subtype antagonist, and CPPG group III antagonist elicited no effects in the expression of the conditioned locomotion response.

The significant difference in the TMT data between cocaine-paired subjects, control, and cocaine-unpaired animals supports our hypothesis that there is an association between the environmental contexts previously paired with cocaine effects (Martin-Iverson and McManus, 1990; Brown and Fibiger, 1992; Brown et al., 1992; Rodríguez-Borrero et al., 2006). One of cocaine’s effect as a psychostimulant is to enhance locomotor activity. On the expression session
Figure 3. Effects of group II blockade within the NAc shell on drug expression session. The parameter used to measure response was the TMT and AD in one hour (measurements are in seconds. 3a A greater locomotor activity was detected in cocaine-paired vehicle subjects than in control vehicle (*p<0.05), and cocaine-unpaired vehicle (*p<0.05). Group II blockade with LY341495 enhanced locomotor activity within all the groups and their counterpart vehicle treated animals, (§§§p<0.001). 3b A greater AD response was observed for the cocaine-paired subject. Group II blockade with LY341495 enhanced locomotor activity within all the groups, cocaine-paired vs. cocaine-paired vehicle treated animals (§§§§p<0.001), treatment within control (§§§§p<0.001) and within cocaine-unpaired ($p<0.05$) subjects.
Fig. 3

A. Group II blockade within the NAc shell during expression session

![Graph showing total move time (s) for different conditions: Control, Paired, Unpaired, with Vehicle and LY341495 treatments.](image)

B. Group II blockade within the NAc shell during expression session

![Graph showing ambulatory distance (cm/60 min) for different conditions: Control, Paired, Unpaired, with Vehicle and LY341495 treatments.](image)
Fig.4. **Effects of mGluR group III blockade within the NAc shell on drug expression session.**

The parameter used to measure response was the TMT and AD in one hour (measurements are in seconds. **4a** Cocaine paired vehicle subjects presented a higher TMT than control (**p<0.01), and cocaine-unpaired vehicle (*p<0.05). **4b** A greater AD response was observed for the cocaine-paired subject, although analyses do not show significantly different results, we were close to reach it (cocaine-paired vs. control p=0.055, cocaine-paired vs. cocaine-unpaired p=0.056). Group III blockade with CPPG did not produce any changes on locomotor activity
Fig. 4

A. Group III blockade within the NAc shell during expression session

B. Group III blockade within the NAc shell during expression session
test, there was a higher response in the conditioned locomotion in the cocaine-paired animals when exposed to only environmental cues. These results suggest that even in the absence of cocaine, the re-exposure to the environmental cues previously paired to cocaine mimic a behavioral response similar to when cocaine is on board.

The present results reveal the presence of classical conditioning between the environmental cues and cocaine effects. This kind of associative learning implies that animals went through a memory process. If this is the case, then this memory seems to be disrupted by microinjection of mGluR5 antagonists within the NAc shell. mGluRs have been previously associated to memory process. Thus, the present mGluR5 findings implicate a role for these receptors in the expression of memories related to environmental cues. Our results showed (figure 2b) a reduction in the locomotion response on the expression of the conditioning in MPEP treated subjects when compared with vehicle animals treated. This finding suggested that mGluR5 within the NAc shell regulated cocaine conditioning effects. Our findings confirmed previous evidence that showed that mGluR5 antagonist reduced cocaine self-administration and cocaine induced reinstatement of drug seeking behavior (Chiamulera et al., 2001; Lee et al., 2005). Previous studies also revealed the role that mGluRs play in memory formation related to associative learning and cocaine effects (Gass and Olive, 2009; Kenny and Markou, 2004; Sidiropoulou et al., 2009; Xie et al., 2010). Moreover, activation of mGluR5 can lead to intracellular cascade of events that could modulate the associative learning of cues previously paired with cocaine (Cleva et al., 2010).

In contrast, blockade of mGluR1 subtype, another member of the mGluR Group I family, with CPCCOEt within the NAc shell, was without effect in the conditioned locomotor response. We expected similar results than the ones obtained with blockade of mGluR5 because they both activate the same intracellular signaling cascade. Previous evidence showed that microinjections of negative allosteric modulators for mGluR1 within the NAc core reduce context-induced reinstatement of cocaine seeking behavior (Xie et al., 2006) and the inhibition of mGluR1 blocked cocaine induced
psychomotor sensitization (Dravolina et al., 2006; Kotlinska and Bochenski, 2011). Our results do not support previous results. However, it also has been demonstrated that acute and chronic cocaine treatment caused a reduction in mGluR1a (plasma membrane bound) within the NAc shell dendrites but the mGluR5 proportion remained intact (Mitrano et al., 2008). A reduction of mGluR1a at the NAc shell can explain why we cannot detect any role of this receptor in the expression of the environmental-elicited cocaine conditioning. Another reason is that even when there is some evidence that support the CPCCOEt dose used (Swanson and Kalivas, 2000), a dose response curve could give a better scenario for the role that this receptor play in the expression of the conditioning.

mGluR2 and 3 are mainly located at the presynaptic level of glutamatergic cells within the NAc, and thus function as autoreceptors that decrease glutamate release (Zheng-Xiong et al., 2002). As autoreceptors, they prevent the formation of (cAMP), by activating a Gi/q protein that inhibits adenylyl cyclase. Group II blockade with LY341495 enhanced the TMT on paired group. At the same time this enhancement can be seen in control and unpaired subjects. This may suggest that these receptors within the NAc shell may play a role in the modulation of locomotion response independently of which exposure animals are receiving (cocaine or saline). Previous studies demonstrated that group II mGluRs modulate motor behavior response. Infusion of LY341495 into the NAc induced an enhancement in the locomotor activity (David and Abraini, 2001). Moreover, knockout mice of mGluR2 subtype presented hyperlocomotion response in different behavior tests. Interestingly, this study demonstrated that these knockout animals showed enhanced cocaine responsiveness (Morishima et al., 2005). In addition, in another experiment, rats were pre-exposed to seven daily injections of cocaine and when challenged the next day with an injection of either saline or the group II mGluR antagonist LY341495, they produced hyper-locomotor activity to LY341495 compared to saline (Yoon et al., 2006). Therefore, our results support the previous data that mGluRs group II plays in locomotion response. If we take into account only the change measured in the cocaine-paired groups we can suggest that this group of receptors negatively
modulated the cocaine rewarding effects. This is because the blockade of these receptors produced the hyperlocomotion activity and exacerbated the expression of environmental-elicited cocaine conditioned locomotion. This means that activation of this receptor and its intracellular cascade counteracted the effects triggered by environmental cues previously associated with cocaine exposure. If that is the case, this receptor within NAc shell could be playing a role of “protection” against cocaine effects. Once again a dose response curve for LY341495 within the NAc shell would give a better understanding of the role of this receptor in the expression of environmental-elicited cocaine conditioned locomotion.

Finally, blockade of group III mGluRs with CPPG had no effect in the expression of the conditioning. There is not much evidence about the function of this group of mGluRs within the NAc shell during cocaine exposure. Behavioral and pharmacological studies reported that intracaudate group III agonist injection, blocked hyperlocomotion induced by acute cocaine exposure and this can be counteracted with a group III antagonist (Mao and Wang, 2001). L-AP4 which is a group III agonist has been found to reduce extracellular DA levels in brain areas such the NAc, whereas MPPG which is a group III antagonist enhanced DA in these regions (Hu et al., 2001; Mao and Wang, 2000; Mao et al., 2013). Thus, by inhibiting DA release, group III receptors may inhibit motor responses to stimulants. Our results do not support previous finding since we did not find any changes in the expression of the conditioning, however, acute and chronic cocaine exposures have different behavioral response and the conditioning paradigms used were not the same. Moreover group III antagonists used were different and they were injected in different brain regions. More studies are necessary to elucidate the role of group III mGluR within the NAc shell during environmental-elicited cocaine conditioning.

In summary, we conclude that mGluRs within the NAc shell can modulate the expression of environmental-elicited cocaine conditioning. Specifically, mGluR5 blockade reduced the locomotor response induced by the exposition of environmental cues previously paired with cocaine. Group II
seems to negatively modulate the conditioning and mGluR1 and Group III had no effect. However, more studies are necessary to characterize mGluRs role, and mechanisms in the expression of cocaine conditioning modulation.
Table1. Blockade of mGluRs within the NAc shell during expression of cocaine locomotion conditioned
<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Receptor</th>
<th>Results</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPCCOEt</td>
<td>mGluR1</td>
<td>No changes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mGluR5</td>
<td>Decreased</td>
<td></td>
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<td></td>
<td>LY341495</td>
<td>Increased</td>
<td>response in all groups</td>
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<tr>
<td></td>
<td>CPGG</td>
<td>No changes</td>
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Chapter III

Metabotropic Glutamate Receptor 5 within NAc Shell Modulates Environment-Elicited Cocaine Conditioning Expression.

Abstract

The mGluRs5 within the NAc have been implicated in the modulation of psychostimulant reward. We hypothesized that blockade of mGluR5 within the NAc shell would impair cocaine conditioning in rats. For this study, animals were implanted with cannulae within the NAc shell, and separate groups were exposed to a multimodal environment within activity chambers that signaled cocaine (cocaine-paired) or saline (controls, cocaine-unpaired) injections. Prior to placing the animals in the chambers, rats received systemic ip injections of saline or cocaine for 10 consecutive sessions. In the test session (D12), animals were exposed to the multimodal environment without any cocaine or saline pre-treatment. Before placing the rats in the chambers, separate groups of animals were infused within the NAc shell with 2.5, 12 or 25nmol/0.5µl/side of 2-methyl-6-(phenylethynyl) pyridine (MPEP), an antagonist of mGluR5 or with vehicle. Blockade of the mGluR5 subtype at a 2.5nmol dose showed no significant difference in either the AD or the vertical plane move time (VPT). In contrast, mGluR5 blockade at 12nmol and 25nmol decreased conditioned locomotion in the cocaine-paired groups. An association of the environmental cues with the effects of cocaine implies the involvement of memory process during the conditioning response. Our results suggest that mGluR5 within the NAc shell could be modulating the expression of memory related to the association of environmental cues with the effects of cocaine. We suggest that mGluR5 could be taking into account to further studies related with cocaine exposure and cocaine addiction treatments.

Introduction

The NAc, localized within the ventral region of the corpus Str has been directly implicated in the mediation of psychostimulant-induced locomotor activity via DA and glutamate neurotransmission (Vezina and Kim, 1999). In addition, the NAc plays a crucial role in behavioral response to rewarding stimuli (Everitt et al., 1999; Ikemoto and Panksepp, 1999; Parkinson et al., 2000), and is associated with the reinforcing and locomotor effects of cocaine (Kobb et al., 1998;
White and Kalivas, 1998). It is subdivided into two different functional sub-regions: the core and the shell. The core region is mainly related to psychomotor functions, because of its connections with basal ganglia and motor output structures (Zaham and Brog, 1992; Zaham and Heimer, 1993). In contrast, the NAc shell is mostly related to emotional processes occurring during psychostimulant reward, because of its connections with limbic structures (Brog et al., 1993; Zahn, 1999).

Within the NAc region, high expressions of mGluRs have a functional interaction with DA terminals (Vezina and Kim, 1999). There are eight mGluR subtypes classified into three groups according to their sequence homology, pharmacology, and second messenger activation (Conn and Pin, 1997). Biochemical studies have revealed that all mGluR groups are found in NAc subregions and play a role in cocaine addiction (Kenny and Markou, 2004; Mitrano et al., 2008).

Group I subtypes (mGluR1 and mGluR5) are coupled to a Gq protein and activate Phospholipase C (Kenny et al., 2004). These receptors are also associated with the intracellular protein Homer (Roche et al., 1999; Xiao et al., 2000; Gasparini and Pin, 2002). It has been demonstrated that after repeated cocaine administration there is an mGluR group I attenuation within the NAc (Swanson et al., 2001). Studies using knockout mice for mGluR5 showed that these animals did not self-administer cocaine, and similar results were observed when these animals were treated with MPEP, an mGluR5 antagonist (Chiamulera et al., 2001; Kenny et al., 2005; Kumaresan et al., 2009). This decrease in cocaine self-administration using MPEP was also reported in rats (Kenny et al., 2004; Paterson and Markou, 2005).

Psychostimulants and other drugs of abuse, including cocaine, can preferentially increase levels of DA within the shell subregion of the NAc, as opposed to its core in rats (Zocchi et al., 2003). Moreover, repeated administration of cocaine increased GABA, glutamate and DA levels within the NAc shell but not the NAc core (Meredith, 1999). The NAc shell has been referred to as a transitional region between the Str and the extended amygdala, (Heimer et al., 1991; Di Chiara et
al., 2006), and is catalogued as the mediator of primary reinforcing effects of drugs, as well as behavioral sensitization (Huang et al., 2011; Pierce and Kumaresan, 2006). It was suggested that changes in the NAc shell induced by cocaine are more often achieved during passive exposure to cocaine (alteration similar to Pavlovian conditioning), while alterations in the NAc core are more predominant during cocaine operant conditioning (Di Chiara 2002; Ben-Shahar et al., 2009). Since the NAc shell is predominantly related to limbic structures and passive cocaine exposure, we hypothesized that this sub-region could also be involved in environment-elicited cocaine conditioning.

It is known that glutamate and its receptors within the NAc shell play differential roles during cocaine treatment. Furthermore, since mGluR5 action is present within limbic regions, it may also be implicated in cocaine conditioned behavioral response, which in turn could be playing a role in the acquisition and expression of a learned response of reward relevance. The present study aims to establish the role of mGluR5 within the NAc shell during expression of environment-elicited cocaine conditioning. We investigated whether blockade of mGluR5 within the NAc shell would disrupt the expression of a conditioned locomotion induced by environmental cues.

Material and Methods

Subjects

Male Sprague Dawley Rats (250-275 g) were used in all experiments. These animals were purchased from Charles Rivers Laboratories (Wilmington, MA). Rats were housed in pairs at the Animal Facility at the University of Puerto Rico, Rio Piedras. Animals were kept at controlled humidity and temperature, on a 12-hour light/dark cycle with food and water available at all times. All procedures were conducted according to the National Institutes of Health Guide for the use of laboratory animals and our animal care institution (IACUC). A total of 79 animals were used for results and statistical analyses.
Cannulae implantation surgery

Surgery was performed as previously described by Rodríguez-Borrero E. et al., 2006. Briefly, all animals were food deprived the day before surgery. On the day of surgery rats were anesthetized with sodium pentobarbital (0.25mg/Kg) (Sigma-Aldrich, MO) and atropine sulfate was administered (0.54mg/Kg) (Phoenix Pharmaceuticals). The 10mm guide cannulae (23 gauges, Small Parts, FL, USA) were implanted using a stereotaxic procedure within the NAc shell (A-P +3.5, M-L ±1.0, D-V -5.3) with the nose bar 5mm above zero. Stainless steel screws (Plastic One, VA, USA) and dental cement (Caribbean Dental Products Corporation, PR) were used to secure the cannulae, and wire stylets were inserted into them. Rats were allowed to recover 5-7 days before starting behavioral experiments.

Microinfusions

For intracerebral microinfusions, we used 12.5 mm injector cannulae (30 gauge, Small Parts, FL, USA). The injector was inserted into the bilateral cannulae in the rat brain and using a microdrive pump (Harvard Apparatus) MPEP 0.5µl/side (Tocris Bioscience, MO) or vehicle 0.5µl/side was administered through polyethylene tubing (Downing, Fisher Scientific Cayey, P.R.). Each rat received only one concentration of MPEP or vehicle and total infusion time was 1 min (min) and 33 seconds followed by a 1 min diffusion period. During that period, the rat was hand-restrained by the investigator. After the microinfusion procedure, injectors were removed and 10 min later the testing session was conducted.

Drugs

Animals were injected ip with either 0.9% sodium chloride solution (Baxter Health Care Corporation, IL) (saline) or 10mg/kg of cocaine-hydrochloride (Sigma-Aldrich, MO) diluted in saline. 2-Methyl-6- (phenylethynyl) pyridine (MPEP) was obtained from Tocris Bioscience, MO.
Doses of MPEP: 2.5, 12 or 25nmol/0.5µl/side were diluted in 50% DMSO (Sigma Aldrich, PA) + 50% Saline (vehicle solution).

**Conditioning methods**

*Activity chamber apparatus:*

TruScan Photobeam Scanning System activity boxes (Coulbourn Instruments, PA USA) were used for the present experiments. The boxes are 41 cm × 41cm × 41 cm on the inside and 53 cm × 53 cm in the base plate. There are 16 photobeams in each box to track animal movement. The boxes are located with visual and olfactory environmental cues in a room different from the room of their home cages. The chambers used for the cocaine-paired group were surrounded by a 61 cm × 61 cm × 61 cm black plexiglass box (visual cue). In addition, California orange oil (Sigma-Aldrich, MO, USA) was released in the room to provide an olfactory cue to the chamber. For the control and cocaine-unpaired groups, the chambers were localized in a different room, surrounded by a 61 x 61 x 61 cm black and white (checkers) plexiglass box and the chamber exposed to a Nutmeg East Indian oil scent (Sigma-Aldrich, MO, USA), an (olfactory cue).

Details of the conditioning protocol used in these experiments are described in Rodríguez-Borrero E. et al. (2006). Briefly, animals were assigned to one of three different conditioning groups: cocaine-paired, control, and cocaine-unpaired.

*Training session*

The training consisted of a conditioning protocol of 10 consecutive days, one session per day for 90 min. Rats were trained during the light phase at approximately the same time on all days. Just before each training session started, rats assigned to the cocaine-paired group were injected i.p. with 10mg/kg of cocaine-hydrochloride. At the end of each training session, animals received an i.p. injection of saline and were returned to their home cages. In contrast, control groups received i.p.
saline injections before and after each training session. The cocaine-unpaired group received i.p. injections of saline before placing them in activity chambers and 10mg/kg of cocaine at the end of the training sessions, after which the animals were returned to their home cages. Animals completed their training sessions in the same activity chamber throughout all the experiments. Two behavioral parameters were measured in all the sessions; (a) AD is measured as distance traveled on the floor plane minus stereotypic movements (b) Vertical Plane Time (VPT) is the total time the animal spent in the vertical plane (z ring).

The control and cocaine-unpaired groups are included to show that just the exposure to a different environment other than their home cage is not sufficient to elicit conditioning. The cocaine-unpaired groups are also included in order to demonstrate that exposure of the animals to cocaine treatment, but not paired to a specific environment, will not trigger conditioning. That group has an equivalent drug history to the cocaine-paired group but lacks the contiguity relationship of environmental cues with cocaine exposure. The experimental relevance of the cocaine-unpaired groups is to show that even though animals receive the same dose of cocaine than the cocaine-paired group, it is not only the drug exposure that induces the locomotor patterns in the activity chambers during the testing session on (D12. It is important to note that our conditioning paradigm is a modified version of the previously published conditioned locomotion protocol developed by Brown and Fibiger, (1992). The paradigm used in the recent study was previously developed and tested in our laboratory as described in Rodriguez-Borrero, et al. 2006, 2010). In order to exclude possible differences in response to the two different environments, all conditioning groups were tested in both the above described environments. As can be seen in Figure 1, no differences in their behavioral profile where observed in all three groups regardless of the environment used.

The establishment of two different rooms to treat the cocaine-paired and control/cocaine-unpaired animals was in order to establish two totally different environments in which just one of
them is related with cocaine. The main objective in our experimental design was to mimic drug use scenarios where drug addicts distinctively discriminate and associate a specific environment cues that signal drug exposure. In order to exclude possible differences in response to the two different environments, all conditioning groups were tested in both environments. In environment 1 the chambers used for the cocaine-paired group were surrounded by black plexiglass box and california orange oil scent. For the control and cocaine-unpaired groups, the chambers were localized in a different room, surrounded by black-and-white plexiglass box (checkers) exposed to a Nutmeg East Indian oil scent. In environment 2 we did a counterbalanced experiment in where we repeated the same conditioning methods, but changed the rooms. During this experiment, control and cocaine-unpaired animals received all the sessions in the room with california orange oil and black boxes. In the other hand, the cocaine-paired group received all the session in the room with nutmeg oil and checkers boxes. As it can be seen in supplementary figure 1, no differences in their behavioral profile were observed in all three groups regardless of the environment used.

We demonstrated that in day 12 the conditioning response of cocaine-paired animals (vehicle treated) is evoked because of the presentation of environmental cues previously paired with cocaine exposure.

*Testing session*

This session was completed two days (D12) after the last training session (day 10 (D10)). On D12, animals were returned to the activity chambers with the environmental cues but without cocaine or saline pre-treatment for a 90 min period. Ten min before placing rats in the chambers, animals received a microinfusion of MPEP (0, 2.5, 12 or 25nmol/0.5µl/side). After completion of the session, the animals were sacrificed, their brains removed and frozen for histology and cannulae verification.

*Histological Analysis*
Frozen brains were cut in 40µm sections using a Leica cryocut cryostat. All sections were mounted in gelatin-coated slides, defatted, and stained (cresyl violet). The location of the cannulae was verified by microscope inspection. If the cannulae were outside of the NAc shell, the animals were excluded from the data analysis.

**Statistical Analysis**

Data are presented as mean±standard error of the mean (SEM) values. For days 1, 5 and 10 (D1, D5, and D10 respectively) data were analyzed using a two way ANOVA to determine: 1) drug conditioning; 2) days effect; and 3) possible interaction. For the test session (D12, expression session), data were analyzed using a two-way ANOVA to determine whether drug conditioning depends on the concentration of MPEP, and whether there is a possible interaction of MPEP microinjection with the conditioning. Post hoc multiple comparison analyses were performed using Tukey test.

**RESULTS**

**Histological analysis**

Placement of the cannulae within the NAc shell was verified using cresyl violet staining. Figure 2 shows a photomicrograph of a representative animal and a schematic representation of rat brains sections with the approximate localization of the cannulae placement. Animals with cannulae outside of the area of study were excluded from analysis. Most of the cannulae were positioned
Figure 1A. Comparison of environmental elicited cocaine conditioning (AD response) between environment 1 and the environment 2. A two way ANOVAs revealed that there is a significant difference between conditioning groups (p<.001). Moreover, it revealed that there is no significant difference between animals tested on environment 1 vs environment 2 in terms of room in where the experiment were performed, neither an interaction between rooms and conditioning. A post hoc showed that cocaine paired vehicle subjects presented a higher AD than control (*** p=0.001), and cocaine-unpaired vehicle subjects (***p<0.001). There is no significant difference between control and cocaine-unpaired subjects. No differences were found between subjects from environment 1 and environment 2 that received the same conditioning.
Fig. 1A  Environmental elicited-cocaine conditioning expression

Ambulatory Distance (cm / 60 min)

- Control
- Cocaine-Paired Conditioning
- Cocaine-Unpaired

Legend:
- Environment 1
- Environment 2

Significance levels:
- *** p < 0.001
Figure 1B. Comparison of environmental elicited cocaine conditioning (VPT response) between environment 1 and the environment 2. A two way ANOVAs revealed that there is a significant difference between conditioning groups (p<.001), and no significant difference between animals tested on environment 1 vs. environment 2 (in terms of room), neither an interaction between rooms and conditioning. A post hoc presented that cocaine paired vehicle subjects showed a higher VPT than control (*** p=0.001), and cocaine-unpaired vehicle animals (***p<0.001). There is no significant difference between control and cocaine-unpaired subjects. No differences were found between subjects from environment 1 and environment 2 that receive the same conditioning.
Fig. 1B

Environmental elicited-cocaine conditioning expression

Vertical Plane Time (s) 60 min

Control  Cocaine-Paired  Cocaine-Unpaired

Conditioning

Environment 1
Environment 2
Figure 2. Photomicrograph of a representative animal and schematic representations of the cannulae placement within NAc shell. Circle symbols represent the approximate bilateral cannulae location within the NAc shell (n=79).
Schematic representation of the cannulae placements within the NAc shell
between +1.70 and +1.20 from bregma. A total of 79 animals were found to have their cannulae placement within the NAc shell.

Cocaine treated animals showed higher locomotor activity than saline treated animals

It is well known that cocaine, as a psychostimulant, enhances locomotor activity. The AD was measured during all days of training to assess the cocaine-induced hyperactivity (Figure 3a). No significant differences for AD were found in the cocaine-paired group throughout the training session. Therefore, we chose to present representative days of this conditioning stage of our protocol (D1, D5 and D10). A two-way ANOVA analysis of the AD showed that there was a significant difference in conditioning (F, [2, 213] = 167.96, p<0.001) and between days (F, [2, 213] = 20.531, p<0.001). Specifically, a post hoc analysis (Tukey Multiple Comparison Test) revealed that there was a significant difference between cocaine-paired animals (n=21) and control animals (n=26) (p<0.001) and between cocaine-paired animals and cocaine-unpaired animals (n=26) (p<0.001) on all days. There was no significant difference between control and cocaine-unpaired animals. For the VPT, a two-way ANOVA analysis showed that there was a significant difference in conditioning (F, [2, 214] = 51.671, p<0.001) and between days (F, [2, 213] = 14.362, p<0.001) with a statistically significant interaction between conditioning and days (F, [4, 214] = 7.987, p<0.001). A post hoc analysis (Figure 3b.) revealed that there was no significant difference between groups on D1. In contrast, behavioral differences were present during D5 and D10 of the training sessions (cocaine-paired animals and control animals (p<0.001); cocaine-paired and cocaine-unpaired animals (p<0.001). No significant difference between control and cocaine-unpaired animals was found.
Figure 3. Cocaine induces hyperactivity during environmental-elicited cocaine conditioning.

The parameter used to measure response was the AD (measurements are in cm) and VPT in one hour (measurements are in seconds). a. Cocaine-paired animals presented a higher locomotor activity (AD) than control and cocaine-unpaired animals (**p<.001) on all days. When comparing cocaine-paired animals between days, there is a higher response on D1 vs. D10 (##p<.01). No significant difference between control and cocaine-unpaired animals were found. Control animals presented a higher AD response on D1 in comparison with D5 (++p<.01) and D10 (+++p<.001). Cocaine-unpaired animals, presented the same response as control b. For VPT, no differences were found between groups on D1. During D5 and D10 of the training sessions, cocaine-paired animals spent more time in the vertical plane than control and cocaine-unpaired animals (**p<0.001). Cocaine-paired animals presented no significant difference between days. No significant difference between control and cocaine-unpaired animals were found. Control animals spent more time in the vertical plane on D1 vs. D5 (###p<.001) and D1 vs. D10 (+++p.01). As control animals, cocaine-unpaired animals presented the same response, D1 vs. D5 (###p<.001) and D1vs. D10 (+++p.01).
Fig. 3a

Locomotor activity during environmental elicited cocaine conditioning

Ambulatory Distance (cm/60 min)

Days

- Control
- Cocaine-Paired
- Cocaine-Unpaired
Fig. 3b

Locomotor activity during environmental elicited cocaine conditioning

Vertical Plane Move Time (s) (60 min)

Days

Control
Cocaine-Paired
Cocaine-Unpaired

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Effects of mGluR5 subtype blockade within the NAc shell

In order to assess the role of mGluR5 during the expression session (D12), animals were microinjected with different doses of MPEP and returned to the activity chambers with the environmental cues but without cocaine or saline injections for a 90 min period (Figure 4a). Statistical analysis of the AD parameter revealed a significant conditioning effect \((F(2, 79) = 17.024, p<0.001)\). Moreover, there is a statistically significant interaction between conditioning and the concentration of MPEP \((F(3, 79) = 2.801, p<0.05)\).

A post hoc analysis demonstrated a higher AD activity of cocaine-paired animals \((n=27)\) when compared with control \((n=26, p<0.001)\) and cocaine-unpaired animals \((n=27, p<0.001)\), but there was no significant difference between control and cocaine-unpaired animals \((p>0.05)\). Comparisons for the conditioning factor within the vehicle showed that the cocaine-paired group \((n=7)\) presented a greater AD over control \((n=6, p<0.001)\) and cocaine-unpaired animals \((n=7, p <0.05)\), indicating that environment-elicited cocaine conditioning was present. The three different doses of MPEP were applied to separate groups of animals. The group that received the lowest dose \((2.5 \text{ nmol of MPEP})\) presented a statistically significant difference between the conditioning factor (cocaine-paired \((n=7)\) and the control \((n=7)\) \(p <0.001\); and cocaine-paired vs. cocaine-unpaired \((n=7)\) \(p<0.01)\), but not between 2.5 nmol of MPEP treated animals and vehicle treated animals within the same group. In contrast, at a higher dose of 12nmol, MPEP significantly decreased locomotor AD activity within the cocaine-paired animals groups (cocaine-paired vehicle \((n=7)\) vs. cocaine-paired 12 nmol MPEP treated animals \((n=6)\) \(p <0.05)\) but not within control groups \((12 \text{ nmol MPEP } n=7)\), and cocaine-unpaired groups \((12\text{nmol MPEP}; n=6)\). The same was seen with the 25nmol MPEP treated animals (cocaine-paired vehicle \((n=7)\), control \((n=6)\) and cocaine-unpaired \((n=7)\). No statistical difference was found between 12 nmol MPEP and 25 nmol MPEP doses of cocaine-paired treated animals vs. control and cocaine-unpaired treated animals with the same dose.
The other behavioral parameter measured was the VPT. A two-way ANOVA of VPT results showed that there was a significant difference in the conditioning factor \( (F (2, 79) = 7.040, p<0.01) \) and a statistically significant interaction between conditioning factor and concentration of MPEP \( (F (2, 79) = 2.332, p<0.05) \). A post hoc analysis (Figure 4b) demonstrated the same pattern of behavior as obtained with AD. For the conditioning factor, results revealed that cocaine-paired animals \((n=27)\) move more at the vertical plane than control animals \((n=26, p<0.01)\) and, than cocaine-unpaired animals \((n=27, p<0.01)\). No difference was found between the control and cocaine-unpaired animals. Similar as for AD, for VPT significant differences in the conditioning factor were found between the vehicle treated animals (cocaine-paired \((n=7)\) vs. control \((n=6)\), \(p<0.001\), and for cocaine-paired vs. cocaine-unpaired \((n=7)\) \(p<0.01\)).

Microinjections of 2.5nmol MPEP into the NAc shell showed that there is a statistically significant difference between the conditioning factor in cocaine-paired animals \((n=7)\) vs. control \((n=7)\) \(p<0.05\), and also in cocaine-paired vs. cocaine-unpaired animals \((n=7)\) \(p<0.05\). However, no difference was found between 2.5 nmol MPEP treated animals and vehicle treated animals within the same group. The higher doses of MPEP (12nmol and 25nmol) reduced the time spent in the vertical plane only in the cocaine-paired groups (cocaine-paired vehicle \((n=7)\) vs. cocaine-paired 12 nmol MPEP treated animals \((n=6)\) \(p<0.01\); cocaine-paired vehicle \((n=7)\) vs. cocaine-paired 25 nmol MPEP treated animals \((n=7)\) \(p<0.01\)) but not within controls groups (12nmol \((n=7)\), 25nmol \((n=6)\)), neither in cocaine-unpaired groups (12nmol MPEP \((n=6)\), 25nmol MPEP \((n=7)\)). The reduction of VPT obtained in the cocaine-paired animals with these two doses, is similar to the time spent in the vertical plane by controls and cocaine-unpaired animals. Thus, higher doses of the mGluR5 antagonist blocked expression of the conditioning, evoking a reduction in the locomotor activity patterns.
Figure 4. Higher doses of MPEP within the NAc shell on drug expression session reduced environmental elicited cocaine conditioning. a. Cocaine paired vehicle subjects presented a higher AD than control (*** p=0.001) and cocaine-unpaired vehicle subjects (*p<0.05). Blockade of mGluR5 with 2.5nmol MPEP resulted in no changes within groups but the differences in conditioning remained. There is a significance difference between cocaine-paired and control animals (### p<0.001) and between cocaine-paired and cocaine-unpaired animals (## p<.01). Blockade of mGluR5 with higher doses (12nmol and 25nmol) of MPEP significantly decreased the locomotor activity of the cocaine-paired animals (cocaine-paired vehicle vs. cocaine-paired 12 nmol MPEP treated animals (§ p< 0.05), cocaine-paired vehicle vs. cocaine-paired 25 nmol treated animals (ϕϕ p< 0.01)) b. VPT showed that cocaine-paired animals presented a higher response (cocaine-paired vehicle vs. control vehicle (**p<0.01); cocaine-paired vehicle vs. cocaine-unpaired vehicle (*p<0.05)). Blockade with 2.5 MPEP nmol resulted in maintenance of the conditioning in comparison with cocaine-paired animals vs. control animals (#p <0.01) but no difference seen in comparison with cocaine-unpaired animals. No changes were presented within the groups. Blockade with 12 and 25 MPEP nmol decreased the VPT only in cocaine-paired treated animals (cocaine-paired vehicle vs. cocaine-paired 12 MPEP nmol treated animals, (§§p<0.01), cocaine-paired vehicle vs. cocaine-paired 25 nmol treated animals, ϕϕ p<0.01)
**Fig. 4a**  
**mGluR5 blockade within NAc shell during expression session**

- Control
- Cocaine-Paired
- Cocaine-Unpaired

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vehicle</th>
<th>2.5nmol MPEP</th>
<th>12nmol MPEP</th>
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**Ambulatory Distance (cm/60 min)**

**Fig. 4b**  
**mGluR5 blockade within NAc shell during expression session**

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<th>Condition</th>
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**Vertical Plane Move Time (s) (60 min)**
Discussion

We investigated the effect of MPEP, an mGluR5 antagonist, within the NAc shell on the expression of a locomotor conditioned response induced by environmental cues. The present results, in which we demonstrated that only the animals from the cocaine-paired group showed increased locomotion when placed in the cocaine-associated environment, are consistent with previous findings (Brown and Fibiger, 1992a; Brown et al., 1992b; Martin-Iverson and McManus, 1990, Rodríguez-Borrero et al., 2006; White et al., 1998). We showed that an association of the environment with cocaine exposure experiences can be elicited in this animal model. Moreover, the presentation of environmental cues (previously paired with cocaine treatment) alone induces a psychostimulant response in the experimental animals not observed in the control or cocaine-unpaired group. Thus, we showed that a strong association of the environment with cocaine exposure can be elicited in this animal model.

Cocaine, as a psychostimulant, enhances locomotor activity in rodents. As expected, during the conditioning training sessions, locomotion (AD) was significantly higher in animals that received cocaine vs. control and unpaired animals. With the other parameter measured, VPT, the locomotion activity was higher in the cocaine-paired group when you compare with control and unpaired group during D5 and D10 but not during D1. This is showed that a strong association of the environment with cocaine exposure can be elicited in this animal model likely due to the exploration factor, since exposure of animals to a new environment causes an increase in locomotor activity due to exploratory behavior (Maldonado-Irizarry and Kelley, 1994). Although different studies indicate that cocaine can induce sensitization (Kalivas et al., 1992; Koff et al., 1994; Pert et al., 1990; Segal and Kuczenski, 1992), during the development of the conditioning in the present experiments no sensitization was observed in the cocaine-paired group. Sensitization is defined as the enhancement of a behavioral response to drugs of abuse as a consequence of repeated administration. In our experiments, cocaine-paired animals did not present this phenomenon.
could be due to several factors such as the low dose of cocaine used and the habituation response of the animal to repeated exposure to the open field procedure. Previous studies support these assumptions. For example, in our published studies and other conditioning experiments in where animals were treated with 10mg/kg of cocaine, no sensitization was observed (Carey et al., 2003; Rodríguez-Borrero et al., 2006). Another explanation for the lack of sensitization effects in our paradigm could be the use of an open-field environment as part of the conditioned stimulus with the locomotor activity as indicative of a psychostimulant-conditioned response (Carey et al., 2003; Ahmed et al., 1996). It is known that locomotor activity is sensitive to habituation effects. Thus, upon repeated exposure to an open field environment, rats become familiar with the environment, and as a consequence become less active (Carey et al., 2005). Since our paradigm combines both a low dose of cocaine and an open field environment, it is possible that the habituation effects generated by repeated testing can reduce the sensitization effects induced by repeated cocaine treatments (Carey et al., 2003).

In the test session (D12), we showed that blockade of mGluR5 within the NAc shell reduced the expression of a locomotor conditioned response of cocaine paired animals induced by environmental cues. Specifically, infusions of 12 and 25 nmol of MPEP within the NAc shell decreased motor activity, suggesting that cocaine conditioning expression is disrupted by the mGluR5 antagonist treatment. This significant reduction was not found in the control or the cocaine-unpaired subjects. Our results imply that these receptors could be modulating the memory processes regulated within the NAc shell for the expression of the association of environmental cues with cocaine effects. Therefore, our findings further support previous experiments that showed that mGluR5 antagonists inhibit cocaine intravenous self-administration and cocaine-induced reinstatement of drug seeking behavior (Backstrom and Hyytia, 2006; Di Ciano et al., 2001; Lee O et al., 2002).
Changes within the NAc shell induced by cocaine are frequently observed during passive exposure to cocaine (similar to Pavlovian conditioning), (Ben-Shahar et al., 2009; Di Chiara, 2002) and our results confirm that mGluR5s within the NAc shell have a role during environment-elicited cocaine conditioning. mGluRs have previously been found to mediate memory formation related to associative learning and cocaine reward (Gass and Olive, 2009; Kenny and Markou, 2004; Sidiropoulou et al., 2009; Xie et al., 2010). In addition, previous studies reported that mGluR5 activation triggers an intracellular cascade that can modulate the associative learning of cues previously paired with cocaine (Cleva et al., 2010). The NAc shell subregion has been implicated with emotional processes based on its connections with limbic structures (Heimer et al., 1997; Zahm et al., 1992; Zahm et al., 1993; Wright et al., 1996), and an extensive body of evidence has demonstrated that limbic structures such as the HPC and the Amyg are required for emotional processes (Buchanan, 2008; LeDoux, 1993; Seymour and Dolan, 2008). Additionally, the NAc shell has been referred to as a transitional region between the Str and the extended Amyg (Heimer et al., 1991). Since the NAc shell has a role in the modulation of the locomotor effects of cocaine, and projects and receives important innervations from the amygdala, it was hypothesized that mGluRs within the NAc region are involved in the cocaine-conditioned behavioral response, and play a role in the learning and expression of that response. The present results suggest that the NAc shell subregion and its limbic glutamatergic connections are important in eliciting a learned conditioned response related to cocaine effects. More specifically, we propose that mGluR5s within the NAc shell are participating in strengthening the associations between environmental stimuli and repeated cocaine exposure.

Different biochemical actions of mGluR5 within the NAc shell can be involved in the modulation of the present cocaine related behaviors reported in this study. One of the proposed cellular mechanisms focuses on interactions between mGluR5 and NMDA receptors (NMDAR). mGluR5s are positively coupled to the NMDAR function, which suggests that activation of mGluR5 can modulate the NMDAR-mediated postsynaptic response. It has also been suggested that
mGluR5 modulates NMDA Long term potentiation (LTP) (Francesconi et al., 2004; Grueter et al., 2007; Jia et al., 1998; Lee et al., 2002). This evidence implicates mGluR5 with learning and memory processes. Furthermore, mGluR5 actively interacts with NMDA function within striatal neurons (Pisani et al., 2001). In our present experiments, it is possible that blockade of mGluR5 decreased NMDA-mediated changes induced by cocaine exposure, therefore supporting the notion that the reported blockade of expression of cocaine conditioning can be a consequence of an mGluR5-NMDAR plasticity modification.

Another mechanism within the NAc shell that may be mediating mGluR5 environment-elicited cocaine conditioning can be through Homer proteins and intracellular cascade activation. The literature supports a role of the Homer family in the different stages of cocaine addiction and in cocaine induced neuronal and behavioral plasticity because of its different patterns of expression during cocaine exposure (Szumlinski et al., 2008; Swanson et al., 2001). Homer is a family of synaptic proteins that play a role in trafficking mGluRs into and out of synapses, connect mGluRs to ionotopic glutamate receptors and regulate mGluR5 intracellular signaling (Brakeman et al., 1997). Thus, it is possible that plasticity related to those processes during environmental-elicited cocaine conditioning could account for the behavioral response found with MPEP in the test session.

In conclusion, mGluR5 within the NAc shell modulates environment-elicited cocaine conditioning expression in rats. We propose that plasticity related with mGluR5 downstream proteins during environment-elicited cocaine conditioning could account for the behavioral response found with blockade of mGluR5 with MPEP. Future studies are needed to characterize the specific mechanisms of action of mGluR5 involved in the expression of environmental-elicited cocaine conditioning. Our results suggest that mGluR5 should be considered as an important target for further studies of cocaine exposure and cocaine addiction.
mGluR5, Homer1b/c and ERK1/2 Expression within NAc Shell is not affected during Environment-Elicited Cocaine Conditioning
Abstract

Glutamate within mesocorticolimbic brain regions has been shown to play an important role during the different stages of cocaine addiction. Therefore, glutamate receptors can be a focus of study as an important target for the development of an effective treatment for drug addiction. Our previous results demonstrated that mGluR5 blockade within the NAc shell disrupts the expression of environmental elicited cocaine conditioning. Thus, a better understanding of the redistribution of mGluR5 within the NAc shell, and the identification of mGluR5 intracellular proteins activation, is relevant in the study of how cocaine exposure affects different aspects of this receptor. The scaffolding Homer 1b/c proteins are of great importance in the trafficking of group I mGluRs. Within the present experiment we aim to establish if expression of the mGluR5 and its scaffolding protein Homer 1b/c are regulated during the different stages of our conditioned locomotion model. In addition, mGluR5 activation initiates an intracellular cascade that involves ERK1/2. We hypothesized that cocaine exposure paired to environmental cues will affect the synaptosomal membrane expression of mGluR5 and Homer 1b/c during the different stages of the cocaine conditioning. Furthermore, we hypothesized that mGluR5 blockade attenuates ERK1/2 activation and this will account for the lower response of cocaine-paired group during expression of the conditioning. For mGluR5 and Homer 1b/c protein levels analyses, separate groups were exposed to a multimodal environment in activity chambers that signaled cocaine (cocaine-paired) or saline (controls, cocaine-unpaired) injections. Prior to placing the animals in the chambers, rats received systemic ip injections of saline or cocaine for 10 consecutive sessions. In the test session (D12), animals were exposed to the multimodal environment without any cocaine or saline pre-treatment. During D1, D5, D10 and D12 animals were sacrificed and immunoblots for the NAc shell subregion were performed. A separate group of animals were used for ERK1/2 studies. Animals were implanted with cannulae within the NAc shell, and subjected to the same conditioning. In the test session, before placing the rats in the chambers, separate groups of animals were infused within the NAc shell with 25nmol/0.5µl/side of MPEP, an antagonist of mGluR5 or with vehicle. For the
experiment 1, results demonstrated that total amounts of mGluR5 and Homer 1b/c protein within the NAc shell were unaffected in any of the treated groups during the different stages of the conditioning. Moreover, we were unable to detect any changes of ERK1/2 phosphorylation levels during expression session.

**Introduction**

The NAc receives excitatory projections from different brain regions such as PFC, HYP and Amyg involved in the modulation of cocaine exposure response. Previous studies established that extracellular glutamate levels within the NAc were modified after acute or chronic cocaine exposure and also during cocaine withdrawal periods (Smith et al., 1995; Pierce et al., 1996; Miguens et al., 2008; Baker et al., 2002; Schmidt et al., 2005). These results implicate that glutamate receptors within the NAc can translate the information that is received from its afferents during the different stages of cocaine exposure/addiction. In addition, neuronal adaptations in the expression and trafficking of glutamate receptors may be emerging in response to extracellular glutamate levels. These adaptations in both pre and postsynaptic glutamate transmission have been implicated in mediating cocaine behavioral response (Pierce et al., 1998; Reid and Berger, 1999; Szumlinski et al., 2004, 2006a; Williams and Steketee, 2004).

It has been demonstrated that mGluR group I expression is altered during cocaine exposure (Swanson et al., 2001; Fourgeaud et al., 2004). These neuroadaptations of mGluR group I expression during cocaine exposure can be mediated by the Homer family of postsynaptic scaffolding proteins (For reviews, Szumlinski et al., 2006b, 2007a). This family of proteins is encoded by three different genes (Homer 1-3) that give rise to constitutively expressed (long isoforms) and, immediate early gene isoforms (For review, de Bartolomeis A and Iasevoli, 2006). The Homer long isoforms contain CC domain that allows them to multimerize, and to interact with glutamate receptors to facilitate their clustering with other proteins and, their localization at cell membrane (Naisbitt et al., 1999; Abe et al., 2003; Rong et al., 2003; Shiraishi et al., 2003 a, b).
Previous studies have demonstrated that cocaine exposure differentially regulated Homer isoforms within brain regions that modulate neuroplasticity in addiction (For review, Szumlinski et al., 2008). Furthermore, there is evidence reporting that Homer long isoforms are involved in regulating glutamate levels and response within the mesocorticolimbic regions during cocaine exposure (Lominac et al., 2005; Szumlinski et al., 2004, 2005a, 2006a). Since Homer long isoform proteins regulate the expression within cell membrane of Group1 mGluRs (Szumlinski et al., 2004, 2005a), the cocaine induced changes in Homer expression can be affecting mGluR5 expression at synaptosomal membrane areas.

mGluR5 activates a Gq protein which initiates the PLC/IP3/DAG cascade (Conn and Pin, 1997). It has been shown that through mGluR5 activation, transcription factor such as CREB and c-Fos were activated (Mao et al., 2002; Yang et al., 2004). This induction of CREB and c-Fos activation can be reversed by MPEP, a mGluR5 antagonist (Mao et al., 2002). In addition, DHPG also enhanced ERK1/2 phosphorylation levels mediated by its conventional PI/Ca2 cascade (Choe and Wang, 2001a, b; Yang et al., 2004; Mao et al., 2005a) and, by mGluR5-Homer1b/c association (Mao et al., 2005a).

ERK1/2 is a member of the MAPK intracellular signaling pathway family (Adams and Sweatt, 2002; Berman and Dudai, 2004). It is known that ERK modulate adaptive processes such as learning, memory, and synaptic plasticity (Sweatt, 2004; Thomas and Huganir, 2004) including those related with drug use, such as cocaine (Valjent et al., 2006; Girault, 2007; for review, Lu et al., 2006). MAPK pathway can be activated by different external signals, leading to a broad cellular response. Cocaine is among these external signals, since acute and repeated cocaine injections increase ERK phosphorylation within mesocorticolimbic system (For review, Lu et al., 2006). NAc ERK activity serves a role in mediating the rewarding effects of psychostimulant during CPP, as inhibition of MEK (an upstream element that activate ERK1/2) before cocaine CPP training, prevent cocaine-induced NAc ERK phosphorylation and, the expression of cocaine CPP (Valjent et
al., 2000). Other experiments support this previous result, and demonstrated that inhibition of ERK phosphorylation will impair and block different stages of CPP (Gerdjikov et al., 2004; Miller and Marshall; 2005; Valjent et al., 2006). These results provided evidence for a role of NAc ERK in memory processes involving cocaine exposure and environmental cues, since systemic treatment that prevents ERK activation during re-exposure to context related with cocaine, blocked the learned behavioral response. ERK and its role in cocaine exposure has also been found in other behavioral procedures like psychomotor sensitization (Girault et al., 2007), and self-administration in which ERK activity modulated the expression of cocaine craving (Lu et al., 2005). All this evidence suggested that ERK have a role in cocaine addiction, including long lasting modulation such synaptic plasticity and, in mediates effects that requires a more rapid response such as expression of CPP (For review, Thomas et al., 2008 ). Thus, mGluR5 could be mediating the activation of ERK during the expression of conditioned locomotion.

Our previous results showed that blockade of the mGluR5 subtype with MPEP, decreased expression of conditioned locomotion in the cocaine-paired groups (Martinez-Rivera et al., 2013). Since in our previous results we demonstrated that mGluR5 has a role during expression of environmental-elicited cocaine conditioning, the present objective aims to establish if mGluR5 and its scaffolding protein Homer1b/c expression are altered within the NAc shell during all the stages of the cocaine locomotion conditioning, including expression stage. It is hypothesized that cocaine modulates Homer 1b/c proteins within the NAc shell during the different stages of our conditioned locomotion protocol, and it is suggested that mGluR5 expression within the NAc shell is affected by Homer 1b/c protein levels. Furthermore, even when there is substantial evidence establishing that mGluR5 activations initiate an intracellular cascade response that involves ERK1/2 activation, it is not clear which mechanism the mGluR5 is utilizing to modulate environmental elicited cocaine conditioning response. We propose that mGluR5 blockade will attenuates ERK1/2 activation and this will account for the lower response of cocaine-paired group during expression of the
conditioning. Our results showed no statistically difference in the expression of mGluR5 neither in Homer1 b/c in any of the stages of environmental elicited cocaine conditioning. In addition, our results showed that after one hour of contextual cues re-exposure, mGluR5 blockade reduces the locomotion response during expression of environmental elicited cocaine conditioning, but at that time ERK1/2 activation was unaffected.

Material and Methods

Subjects

Male Sprague Dawley Rats (250-275 g) were used in this experiment. These animals were purchased from Charles Rivers Laboratories (Wilmington, MA).

Drugs

Animals were injected ip with either 0.9% sodium chloride solution (Baxter Health Care Corporation, IL) (saline) or 10mg/kg of cocaine-hydrochloride (Sigma-Aldrich, MO) diluted in saline.

Conditioning methods

Animals were subjected to one of three different groups: controls, cocaine-paired, and cocaine-unpaired. The same conditioning methods previously described (chapter I and chapter II) were used to perform the studies in this chapter.

Experiment 1; mGluR5 and Homer 1b/c expression

During the conditioning, animals from each one of the groups were sacrificed at D1, D5, D10 or at the end of the conditioning (D12). After the session was completed, the animals were sacrificed, brain were obtained and frozen for mGluR5 and Homer 1b/c immunoblots analysis.

Protein Extraction

Animals from each group (control, cocaine-paired, cocaine-unpaired) were sacrificed by decapitation. A naïve group of animals was handled, maintained at the animal house facilities and sacrificed the same day than the experimental groups. After sacrifice, brains were removed and
frozen immediately on dry ice after test session completion. NAc shell samples were obtained from chilled coronal brain slices (1.2-2.2 mm anterior to bregma) using a 12-gauge blunt-end needle attached to a 10cc syringe. Pools of 4 animals were prepared for each group.

The homogenization of the NAc shell, were done as described by biochemical fractionation (Ghasemzadeh, et al. 2009). Tissue pellets were homogenized with Dounce homogenizers with loose and tight pestles. Homogenize tissue (H) in ice-cold TEVP buffer containing: 20 mm Tris HCl pH 8.0, 10 mM NaF, 2 mM Sodium Orthovanadate, 2 mM EGTA, 2mM EDTA and 640 mM sucrose and centrifuged at 1000 × g to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 10,000 × g to obtain a crude synaptosomal fraction (P2) and subsequently was lysed hypo-osmotically and centrifuged at 25,000 × g to pellet a synaptosomal membrane fraction (LP1). LP1 is a fraction enriched in postsynaptic density and it was resuspended in TEVP buffer. The H (total cell protein) and LP1 fraction were solubilized with the addition of SDS to 1% (v/v) and were used for protein concentration measurement and immunoblot analyses. All fractions were kept at -80.

**Immunoblots**

Western blot analyses for mGluR5 and Homer 1b/c were performed using 10% Acrylamide/Bis SDS-PAGE gels. Equivalents amounts of H and LP1 fraction of each sample were loaded (10ug). A protein molecular weight standard (Precision Plus Protein All Blue, BioRad) was included in a gel lane. Samples were then transferred to nitrocellulose membrane (membranes were first equilibrated for 10-15 min in Towbin buffer) using the BioRad Mini Protean System at 4°C for 1:00 hour at 100V. Protein transfer was verified by soaking membrane in Ponceau S (vegetable stain for total protein visualization, BioRad). Membranes were blocked for 2 hours (blocking buffer; TBS containing 3% non-fat dry milk) at room temperature and then incubated overnight at 4°C with Homer 1b/c (Santa Cruz, biotechnology antibodies) or anti-mGluR5 (Millipore, MA) primary antibody at a concentration of 1/3000 μl in antibody buffer (blocking buffer containing tween-20, 50ul/100 ml). Next day blots were washed with antibody buffer, and incubated with
HRP-conjugated secondary antibody for 90 min at room temperature. Membranes were washed (TBS-Tween 20, 25µl/100ml) and immunolabeling was visualized with enhanced chemiluminescence (ECL).

**Experiment 2; ERK and pERK expression**

Animals used for ERK and pERK immunoblots analyses went through cannulae implantation surgery. Briefly, as previously described (chapter I and II), rats were anesthetized with sodium pentobarbital (0.25mg/Kg) and atropine sulfate was administered (0.54mg/Kg). The 10mm guide cannulae were implanted within the NAc shell (A-P +3.5, M-L ±1.0, D-V -5.3, rats were placed with the nose bar 5.0 mm above interaural zero). After at least 5 days of recovery, animals went through the behavioral conditioning previously described (chapter I and II). For microinfusions, 12.5 mm injectors were inserted into the bilateral cannulae in the rat’s brain. MPEP or vehicle (0.5µl/side) was microinjected through polyethylene tubing with a microdrive pump. Each rat received only one concentration of MPEP (25nmol) or vehicle and total infusion time was 1 min and 33 seconds, followed by a 1 min diffusion period. After the microinfusion procedure, injectors were removed and 10 min later the testing session was conducted. At the end of the session, the animals were sacrificed, their brains removed and frozen for histology and cannulae verification or immunoblots analysis.

**Protein Extraction**

Animals from each group (control-vehicle, control-MPEP, cocaine-paired-vehicle, cocaine-paired-MPEP, cocaine-unpaired-vehicle and cocaine-unpaired-MPEP) were sacrificed by decapitation. A naïve group of animals was handled, maintained at the animal house facilities and sacrificed the same day than the experimental groups. After sacrifice, brains were removed and frozen immediately on dry ice after test session completion. NAc shell samples were obtained from chilled coronal brain slices (1.2-2.2 mm anterior to bregma) using a 12-gauge blunt- end needle attached to a 10cc syringe. Pools of 4 animals were prepared for each group. Tissue was then
homogenized with a polytron homogenizer in extraction buffer (20mM Tris base pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM PMSF, 5mM Sodium Pyrophosphate, 10mM β-glycerolphosphate, 30mM Sodium Fluoride). The volume of extraction buffer added to the samples was determined by the following equation: \( \text{Volume of buffer (ml)} = \text{weight of sample (g)} \times 5 \)

The homogenates were centrifuged in a refrigerated ultracentrifuge at 4°C for 10 min at a velocity of 14,000 RPM. Supernatants (cytosolic fraction) were removed and stored at -80 until further manipulation. Protein concentration was determined using the Bradford Method.

**Immunoblots**

Western blot analyses for ERK1/2, and pERK1/2 were performed using 10% percent Acrylamide/Bis SDS-PAGE gels. Equivalents amounts of total protein from the cytosolic fraction of each sample were loaded (12μg). A protein molecular weight standard (Precision Plus Protein All Blue, BioRad) was included in a gel lane. Samples were then transfer to nitrocellulose membrane (membranes were first equilibrate for 10-15 min in Towbin buffer) using the BioRad Mini Protein System at 4°C for 1:00 hour at 100V. Protein transfer were verifying by soaking membrane in Ponceau S (vegetable stain for total protein visualization, BioRad). Blots were incubated with Odyssey Blocking Buffer (PBS) (Li-cor Bioscience) for 1:30 hour at room temperature in the shaker. After this step membranes were incubated in the shaker with the primary antibodies in odyssey blocking buffer, ERK1/2 (1/1133) (Cell Signaling, #9102), or pERK1/2 (1/1000) (Cell Signaling, #9101) overnight at 4°C. Next day, blots were washed two times each for 10 min with washing solution (1mM Tris, 150nm NaCl, pH 7.4 and .025% v/v Tween-20). After that they were incubated with the secondary antibody, IRDye 800CW Donkey anti Rabbit (1/15000) (Li-cor Bioscience, #926-32213) for 1:30 at room temperature in western blot dark incubation boxes. Membranes were washed four times with washing buffer, 5 min each (the last wash was performed in TBS without tween) and exposed at the Oddysey imager Scanner. Blots were left in TBS at 4°C until further use. In order to normalize the ERK1/2, and pERK1/2 membranes were reprobed with
GAPDH. Membranes were first block in Odyssey blocking buffer for 1:00 hour at room temperature. After this step membranes were incubated in the shaker with the GAPDH (1/3000, Immuno Chemical Clone 6C5) primary antibody in odyssey blocking buffer, over night at 4°C. Next day, blots were washed two times each for 10 min with washing buffer and incubated with the secondary antibody, IRDye 680LT Donkey anti Mouse (Li-cor Bioscience, #926-68022) for 1:30 at room temperature in western blot dark incubation boxes. Membranes were treated as previously described. For further antibodies incubations membranes were washed, with agitation (70 RPM) in 37°C for 15 min in 2X stripping buffer (New Blot Nitro Stripping Buffer (5x), Li-cor Bioscience, # 928-40030). A second stripping washed was performed, for 8 min with 5X stripping buffer. Membranes were washed with PBS 3 times for 5 min (after each stripping washed) blots were verifying for confirmation of antibodies removal. Then, membranes were treated as previously described for further antibodies reprobal.

Statistical Analysis

Data are presented as SEM values. In the experiment 1, for behavioral response and for protein expression, data was analyzed using a one way ANOVA to determine if protein expression depended on the conditioning they received. Post-hoc multiple comparison analysis was performed using Tukey test. For behavioral response in the experiment 2, data was analyzed using a two way analysis of variance (ANOVA) to determine whether drug conditioning depends on MPEP treatment, and whether there is a possible interaction of MPEP microinjection with the conditioning. Post-hoc multiple comparison analyses were performed using Tukey test. For western blot the same statistical analysis was used to determine if protein expression is affected by conditioning and MPEP treatment.

Results

Experiment 1
Behavioral response during all stages of environmental elicited cocaine conditioning: cocaine-paired animals showed higher locomotor response

The AD was measured during all stages of training to assess the cocaine-induced hyperactivity (Figure 1a-c) and to assess the environmental elicited-cocaine conditioning locomotion during expression session (Figure 1d). The days that are represented are D1 (acute cocaine injection), D5 (early acquisition of our conditioning), D10 (last day of cocaine ip injection, late acquisition) and D12 (expression test of the conditioning). Results for acute cocaine injection presented a robust higher locomotors response for the cocaine-paired animals (F, [2, 91] = 153.9, p<0.001) with a post hoc analyses showing a p<0.001 for cocaine-paired vs. control animals and the same for the comparison between cocaine-paired and cocaine-unpaired animals (Figure 1A). As in D1, during the fifth and tenth day of the conditioning, locomotors response was higher in cocaine paired animals (D5=F, [2, 71] = 46.76, p<0.001, D10=F, [2, 44] = 22.66, p<0.001) (Figure 1B-C). For D12, a one-way ANOVA analysis of the AD showed that there was a significant difference in conditioning during expression session (F, [2, 32] = 6.837, p<0.01). Specifically, a post hoc analysis (Tukey Multiple Comparison Test)
Figure 1. Behavioral locomotion response (AD) during all stages of environmental elicited cocaine conditioned locomotion. The parameter used to measure response was the AD (measurements are in cm) in one hour (measurements are in seconds). A-C Cocaine-paired animals showed a higher locomotor activity (AD) than control and cocaine-unpaired animals (D1, D5 and D10 ***p<.001) during all days of training session. No significant difference between control and cocaine-unpaired animals were found. Behavioral results data for D1, D5, D10 and D12 were obtained from all animals used for immunoblots. Behavioral data of a total of 92 animals were used for D1 (control n = 33, cocaine-paired = 28, cocaine-unpaired = 31). For D5 the amount of animals for control n = 25, cocaine-paired n=28, cocaine-unpaired n=19, and for D10 control n=16, cocaine-paired n=18 and cocaine-unpaired n=11. The discrepancy in the numbers was because in some days data was not recuperated because of technical issues. However, since animals went through all the processes of the experiment they still were used for immunoblots. D. For the expression session cocaine-paired (n=10) animals showed a higher response than control (n=8) (*p<.05) and unpaired animals (n=8) (**p<.01).
revealed that there was a significant difference between cocaine-paired animals \((n=10)\) and control animals \((n=8)\) \((p< 0.05)\) and between cocaine-paired animals and cocaine-unpaired animals \((n=8)\) \((p< 0.01)\) during expression session. There was no significant difference between control and cocaine-unpaired animals (figure 1D).

**Total mGluR5 and Homer 1b/c protein expression within the NAc shell during all stages of environmental elicited cocaine conditioning**

mGluR5 and Homer 1b/c expression was assessed during D1, D5, D10 and D12 of our locomotion conditioning protocol. As depicted in figure 2 and 3 total mGluR5 and Homer 1b/c protein expression within NAc shell was unaltered during all days of testing. During D1, animals received a saline or cocaine injection, immediately after the injection they were exposed to environmental cues in the open field arena for one hour. After the conclusion of the experiments, animals were sacrificed and protein was obtained for further immunoblots analyses. Acute cocaine injection did not affect the expression of total mGluR5 and Homer 1b/c proteins during D1. The same procedure was performed in order to obtain NAc shell mGluR5 and Homer 1b/c proteins for D5 and D10. During D12 (expression of the conditioning session), the environmental cues previously paired with cocaine (or saline) failed to induce any changes on total mGluR5 or Homer 1b/c expression.

**Membranal mGluR5 and Homer 1b/c protein expression within the NAc shell during all stages of environmental elicited cocaine conditioning**

The LP1 fraction was used to assess the synaptosomal membrane mGlur5 and Homer 1b/c protein expression within the NAc shell (Figure 4 and 5). During D1 synaptosomal mgluR5 and Homer 1b/c levels within the NAc shell were enhanced however, data did not reach significant differences (Fig. 4A and 5A). During D5 and D10 there were no protein expression differences between groups. Chronic cocaine exposure
Figure 2. Total mGluR5 protein expression within the NAc shell during all stages of environmental elicited cocaine conditioned locomotion. mGluR5 proteins levels were unaffected during any of the stages of our conditioned locomotion. A pool of 4-5 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs. C=control, P= cocaine-paired and U= cocaine-unpaired.
Figure 3. Total Homer 1b/c expression within the NAc shell during all stages of environmental elicited cocaine conditioned locomotion. Homer 1b/c proteins levels were unaffected during any of the stages of our conditioned locomotion. A pool of 4-5 animals per group was submitted to immunoblots test. Two different groups/pools were assessed and the overall results are presented in the graphs. C=control, P= cocaine-paired and U= cocaine-unpaired.
Figure 4. Membranal mGluR5 protein expression within the NAc shell during all stages of environmental elicited cocaine conditioned locomotion. mGluR5 proteins levels were unaffected during any of the stages of our locomotion conditioning. A pool of 4-5 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs. C=control, P=cocaine-paired and U=cocaine-unpaired.
Figure 5. Membranal Homer 1b/c protein expression within the NAc shell during all stages of environmental elicited cocaine conditioned locomotion. Homer 1b/c proteins levels were unaffected during any of the stages of our conditioned locomotion. A pool of 4-5 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs. C=control, P= cocaine-paired and U= cocaine-unpaired.
(D5 and D10) was unable to alter these proteins levels. Furthermore, pairing of environmental cues with cocaine or saline exposure was unable to alter these proteins right after animals were exposed to environmental cues for one hour (Figure 4D and 5D).

**Experiment 2**

**Histological analysis**

Placement of the cannulae within the NAc shell was verified using cresyl violet staining. Figure 6 shows a schematic representation of rat brains sections with the approximate localization of the cannulae placement. Animals with cannulae outside of the area of study were excluded from analysis. A total of 24 animals were used for analysis.

**mGluR5 blockade within the NAc shell attenuates expression of locomotion conditioning after one hour of contextual cues re-exposure**

Animals were microinjected with vehicle or MPEP and returned to the activity chambers with the environmental cues but without cocaine or saline injections for a 60 min period (figure 7). Results confirmed our previous data in where there is a higher response for the cocaine-paired group during re-exposure to the context cue, and this response is attenuated with MPEP. Statistical analysis of the AD parameter revealed a significant conditioning effect \( F(2, 63) = 3.297, p<0.05 \). A post hoc analysis demonstrated a higher AD activity of cocaine-paired animals \( n=12 \) when compared with control \( n=9, p<.05 \) or with cocaine-unpaired \( n=12, p<.05 \) treated with vehicle. In addition, within cocaine-paired animals MPEP treatment attenuates locomotion response \( p<.05, n=12 \) vehicle, \( n=12 \) MPEP).

**mGluR5 blockade within the NAc shell does not attenuates expression of ERK1/2 phosphorylation levels**
Figure 6. Schematic representations of the cannulae placement within the NAc shell. Circles symbols represent the approximate bilateral cannulae location within the NAc shell (n=24). Injector sites may appear fewer than the reported number of rats because of overlap of placements.
Fig. 6  Schematic Representation of the cannulae placements within NAc shell
Figure 7. MPEP within the NAc shell on drug expression session reduced environmental elicited cocaine conditioned locomotion. a. Cocaine paired vehicle subjects showed a higher AD than control (#p<0.05) and cocaine-unpaired vehicle subjects (#p<0.05). Blockade of mGluR5 with 25nmol of MPEP significantly decreased the locomotor activity of the cocaine-paired animals (cocaine-paired vehicle vs. cocaine-paired, *p< 0.05). No differences were obtained within control animals (control vehicle n=9, control MPEP n=9) or within cocaine-unpaired group (cocaine-unpaired vehicle n=12, cocaine-unpaired MPEP n=9). Graph represents the behavioral response of the animals that were used for cannulae verification and for protein expression analyses.
mGluR5 blockade within the NAc shell during expression session
We decided to investigate if mGluR5 blockade within the NAc shell disrupted the expression of environmental elicited cocaine conditioning via ERK1/2 activation. Western blot analysis from the NAc shell after test session (D12) revealed that either inactive (ERK1/2) or active ERK1/2 (pERK) were affected during the expression session (figure 8-9). In terms of pERK44/ERK42 ratio, no changes were obtained neither a tendency (figure 10). In contrast, pERK44/ERK44 ratio showed a tendency of enhancement within the cocaine-paired vehicle treated group, and within the control animals MPEP treatment diminished the ratio. Within unpaired animals no changes neither a tendency was detected. When we compared the levels of pERK42/Total ERK (T-ERK) or pERK44/T-ERK no difference was detected within the different groups (Figure 11).

**Discussion**

We investigated how mGluR5 and Homer 1b/c proteins expression within the NAc shell was affected as consequence of cocaine exposure paired with environmental cues. First of all, as expected, during the conditioning training sessions, locomotion was significantly higher in animals that received cocaine vs. control and unpaired animals. In addition, with the behavioral analysis we were able to confirm our previous results where an association between the environment and cocaine exposure experiences can be elicited in this animal model (Martinez-Rivera et al., 2013).

mGluRs group I interactions with the different isoforms of Homer 1 protein affects different processes, such as cell signaling, plasticity and behavior response (For review, Gao et al., 2013). Homer 1 scaffolding protein and mGluR5 levels of expression have been extensively studied during different stages of cocaine addiction. However, most of the studies have been focused in how these proteins are affected after long withdrawal periods of cocaine exposure, specifically with sensitization or self-administration paradigms. Even when we did not find any significant change in the expression of mGluR5 and Homer 1b/c protein, our results demonstrated for the first time the
Figure 8. ERK protein levels within the NAc shell during the drug expression session of environmental elicted cocaine conditioned locomotion. A. Representative immunoblots of ERK42, ERK 44 and loading control GAPDH within the NAc shell during the drug expression session, N=Naïve, CV=Control Vehicle, CM=Control MPEP, PV= Cocaine-paired vehicle, PM= Cocaine-paired MPEP, UV= Cocaine-Unpaired Vehicle and, UM= Cocaine-Unpaired MPEP. B-D. Representative graphs for the total levels of ERK42 and ERK44 (Total-ERK (T-ERK)), ERK42 and ERK44 respectively. A pool of 4 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs (a total of 8 animals per each graph bar).
ERK protein levels within the NAc shell during expression of environmental elicited cocaine conditioning

**A.**

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N  CV  CM  PV  PM  UV  UM

**B.**

T-ERK expression within NAc shell during expression session

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**C.**

ERK 42 expression within NAc shell during expression session

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**D.**

ERK 44 expression within NAc shell during expression session

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**Fig. 8**
Figure 9. pERK protein levels within the NAc shell during the expression of environmental elicited cocaine conditioned locomotion. A. Representative immunoblots of pERK42, pERK 44 and loading control GAPDH within the NAc shell during the expression session N=Naïve, CV=Control Vehicle, CM=Control MPEP, PV= Cocaine-paired vehicle, PM= Cocaine-paired MPEP, UV= Cocaine-Unpaired Vehicle and, UM= Cocaine-Unpaired MPEP. B-D. Representative graphs for the total levels of pERK (T-pERK), pERK42 and pERK44 respectively. A pool of 4 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs (a total of 8 animals per each graph bar).
**Fig. 9**

**pERK protein levels within the NAc shell during expression of environmental elicited cocaine conditioning**

**A.** pERK 44 and pERK 42

**B.** T-pERK expression within NAc shell during expression session

**C.** pERK 42 expression within NAc shell during expression session

**D.** pERK 44 expression within NAc shell during expression session
Figure 10. ERK and pERK protein levels within the NAc shell during the expression of environmental elicited cocaine conditioned locomotion. A-C Representative graphs for ratio of pERK 42/ERK42, pERK44/ERK44 and, for the total levels (T-pERK/T-ERK). A pool of 4 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs (a total of 8 animals per each graph bar).
ERK and pERK protein levels within the NAc shell during expression of environmental elicited cocaine conditioning

A. pERK42/ERK42

B. pERK44/ERK44

C. T-pERK/ T-ERK
Figure 11. ERK and pERK protein levels within the NAc shell during the drug expression of environmental elicited cocaine conditioned locomotion. A-B. Representative graphs for ratio of pERK 42/T-ERK and pERK44/T-ERK. A pool of 4 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs (a total of 8 animals per each graph bar).
Fig. 11

A. pERK42/T-ERK expression within the NAc shell during expression session

B. pERK44/T-ERK expression within the NAc shell during expression session
patterns of expression of these two proteins within the NAc shell during development of cocaine locomotion conditioned.

We did not reach a statistical significant difference during acute cocaine ip injection and subsequently exposure of environmental cues, however we detected a tendency of enhancement of mGluR5 and Homer1b/c proteins (1 hour after cocaine injection and contextual cue exposure) on D1. In Support to our results (no changes), Mao & Wang (2001), reported no changes in mGluR5 mRNA expression within ventral Str (NAc) at 3 hours after acute amphetamine administration (Mao and Wang, 2001). In addition, 45 min and 24 hours after an acute cocaine injection (30mg/kg) no changes were detected of mGluR5 in the plasma membrane and dendrites in the NAc shell (Mitrano et al., 2008). Contradictory to these results, another study reported that acute cocaine injection induced an enhancement of total Homer 1b/c within the NAc shell but a decrease in total levels of mGluR5 expression after 24 hours (Forgeaud et al., 2004). An induction of total Homer 1b/c was also observed predominantly in the purkinje cells of the cerebellum after 15 hours of acute injection (Dietrich et al., 2007). In addition, researchers report an enhancement of Homer 1b/c at 24 or 15 hours after cocaine injection. It is possible that the tendency in Homer 1b/c enhancement that we obtained, will reach significant difference if it’s measured in a later time point. In addition, in our results the tendency of enhancement is more obvious in mGluR5, than in Homer 1b/c. The literature suggests that Homer 1b/c have a dual role during the association with mGluR5. Homer 1b/c can couple with mGluR5 at membrane to interact with other proteins thus, promoting the activation of intracellular signals. In addition, there is evidence showing that this interaction between Homer 1b/c and mGlur5 can arrest the receptor in the endoplasmic reticulum (ER) (Roche et al., 1999; Ango et al., 2002). In contrast, Homer 1a can disrupt mGluR5-Homer 1b/c interactions and function as a dominant negative (prevent Homer 1b/c-mGluR interaction and downstream effects). Thus, suggesting that Homer 1a will promote expression of mGluR5 at the membrane instead of being arrested in the ER (Roche et al., 1999; Ango et al., 2002). Different studies suggest
an enhancement of Homer 1a during acute cocaine exposure. For example, Homer 1a mRNA levels were elevated after 1 hour post injection of cocaine and this elevation remained until 2 hours, but at 6 hours levels were as control (Ghasemzadeh, 2009b). Similar results were reported for protein levels in where total Homer1a expression was elevated within dorsal and ventral Str cells after 2 hours of cocaine injection but no differences were obtained for Homer 1b/c levels (Zhang et al., 2007). Although there is not a clear scenario in how these Homer proteins are modulating traffic in and out of the ER and promoting their expression in the plasmatic membrane. We suggest that Homer 1a is enhanced after acute cocaine injection (as the literature suggests), and this accounts for mGluR5 enhancement tendency one hour after cocaine injection.

Acute cocaine treatment, results in an enhancement of glutamate levels within the NAc (Smith et al., 1995; Reid et al., 1996). This effect leads to the internalization of AMPA GluR1- and GluR2-containing receptors (Boudreau and Wolf, 2005; Boudreau et al., 2007). Taking into account that we did not obtain any significant difference in mGluR5 levels, it could be possible that the inducement of glutamate levels produced by acute cocaine was not high enough to affect group I mGluRs trafficking. In the other hand, if we consider the tendency of enhancement of mGluR5, it could be possible that the cell is trying to maintain a balance in their glutamate response. Thus, since there is a rapid AMPA receptors internalization, glutamate effects still can be translated with the unaffected levels of group I mGluRs or with a transient mGluR5 enhancement.

Little is known about mGluR5 and Homer 1b/c protein levels during continuous cocaine exposure (early and late acquisition). We did not detect any difference in mGluR5 and Homer 1b/c during D5 and D10 of our cocaine conditioned locomotion protocol. It was reported that after 15 hours of the last 10 consecutive cocaine injections, there was an enhancement of total Homer 1b/c within the purkinje cells in the cerebellum (Dietrich et al., 2007). An amphetamine study showed that after 3 hours of continuous amphetamine exposure, mGluR5 mRNA was reduced within cultures cells of the Str (Mao and Wang, 2001). However, not much relation can be established
between the studies, because the protocols, brain region, drugs and, time after drug injection vary in comparison with our study. In terms of D12 protein expression, we can establish some comparison with studies of withdrawal periods. We found no changes on mgluR5, neither in Homer 1b/c during D12. We can say that this day can account for 48 hours of withdrawal of cocaine injections. At one day of withdrawal from sensitization protocol, there were no changes on total or synaptosomal protein levels of mGluR5 or Homer 1b/c within the NAc shell (Ghasemzadeh et al., 2009c). In self-administration of cocaine, it was found that after 24 hours of withdrawal, Homer 1b/c levels were reduced but not at 2 weeks of withdrawal (For review, Szumlinski et al., 2008). Most of the data for withdrawal periods after cocaine exposure reported changes after longs periods of the last cocaine exposure. A reduction of mGluR5 mRNA after 7 days (or longer) of amphetamine withdrawal periods were reported (Mao and Wang, 2001). In line with this previous result, extinction of cocaine seeking behavior results in lower levels of synaptosomal mGluR5 in the NAc shell (Ghasemzadeh et al., 2009). In addition, 3 weeks of withdrawal from repeated cocaine administration downregulated total Homer1b/c within the NAc shell but total mGluR5 protein levels were unaffected (Ary and Szumlinski, 2007). However, mGluR5 and Homer 1b/c protein levels were enhanced after 21 days of sensitization withdrawal (Ghasemzadeh, 2009c). Thus, different pattern of expression for these two proteins can occur depending in what kind of cocaine treatment they received, cell fraction analyzed, and how many withdrawal days had passed. At least we can assume that at two days of withdrawal from cocaine is not enough to detect changes in these two proteins. It is possible that with longer withdrawal periods, we will be able to obtain changes in mGluR5 and Homer 1b/c proteins levels.

During D12 of our cocaine induced locomotion conditioning protocol, we need to take into account that it involves learning and memory processes since cocaine-paired animals are re-exposed to the environment previously paired with cocaine. Recent data demonstrated that disrupting mGluR5-Homer 1b/c interactions within NAc core, inhibited cocaine cue induced reinstatement,
but mGluR5 total and surface expression is not affected, neither total Homer 1b/c levels (Wang et al., 2013). Though, we can suggest that re-exposure to environmental cues previously paired with cocaine does not affect protein expression of these two proteins but, it is possible that disruption of mGluR5-Homer 1b/c interactions will disrupt expression of environmental elicited cocaine locomotion conditioning.

Our previous finding demonstrated that blockade of mGluR5 within the NAc shell blocked the expression of environmental elicited cocaine conditioned locomotion. We decided to investigate if mGluR5-ERK1/2 activation was driving the expression of this conditioning. Our results demonstrated that active and inactive levels of ERK1/2 were unaltered after expression of the conditioning. However, cocaine-paired animals presented a mild tendency of pERK1/ERK1 (pERK44) enhancement after re-exposure to environmental cues previously paired with cocaine and this was unaffected by MPEP treatment after one hour of re-exposure.

Biochemical evidence support the role of mGluR5 in activating ERK1/2 intracellular cascade, and MPEP disrupting this activation (Mao et al., 2002; Schroeder et al., 2008). Surprisingly, no differences in ERK1/2 levels were obtained between animals treated with vehicle or MPEP in our experiment. In addition, re-exposure to contextual cues previously paired with cocaine was not enough to induce pERK within NAc shell in cocaine-paired animals. Few studies support our results, however Tropea et.al., (2008) also reported no changes in pERK1/2 within NAc after 20 min of re-exposure to contextual cues previously paired with cocaine (Thomas et al., 2008). We believe that different factors can account for our results. First, phosphorylation of ERK after acute cocaine exposure was enhanced within NAc shell at 5 min, at 10 min most of this pERK was translocated to the nucleus and, at 20 min the cocaine-induced ERK phosphorylation was dramatically reduced (Besson et al., 2000). These, finding suggest that cocaine exposure induced a rapid but transient ERK activation within the NAc shell. In addition, the activation of ERK after acute cocaine injection seems to be driven by D1 receptor, since blockade of this receptor before
cocaine injection abolished the cocaine-induced pERK (Besson et al., 2000). Not only D1R modify ERK activation, NMDAR blockade completely abolished the cocaine-induced pERK within the NAc without affecting hyperlocomotion response induced by acute cocaine (Besson et al., 2000). In another experiment they found that CPP enhanced ERK activation, however the test only lasted for 18 min, a difference in time that should be taking into consideration, since our test session lasted for one hour. In these animals, a second test was performed, 24 hours before a second test session, animals received SL3627 (pERK inhibitor) accompanied with a cocaine injection and CPP was abolished (Valjent et al., 2006b). These findings suggest that ERK activation is necessary for CPP expression. Interestingly, if these animals received a saline injection (instead of cocaine) in parallel with SL327, CPP expression was not inhibited (Valjent et al., 2006b). Three main points can be obtained from these experiments, first it is possible that we were unable to detect any changes in ERK activation because at one hour after re-exposure to contextual cues previously paired with cocaine, pERK was already attenuated or for instance translocated to the nucleus (we just measured the pERK within cytoplasmic cell fraction). Second, although MPEP was previously showed to reduce ERK1/2 activation, it is possible that in our environmental elicited cocaine conditioning paradigm other receptors are also driving pERK expression within the NAc shell, such as D1R and NMDAR (as suggested by others; Berhow et al., 1996; Valjent et al., 2000, 2004; Berman and Dudai, 2004; Schroeder et al., 2008) an explanation of why MPEP treated animals did not reduce ERK phosphorylation after one hour. Third, ERK1/2 activation may have a role in modulating the expression of CPP but exposure to drug-associated environment was not sufficient for SL327 (ERK activator inhibitor) to abolish CPP (Besson et al., 2000). It is possible that in our environmental elicited-cocaine conditioning pERK was unaltered because is not the principal protein driving expression when contextual cues are present.

Another explanation for the lack of changes in ERK activation in the cocaine-paired group is that continuous cocaine exposure is not as efficient as acute cocaine in inducing pERK. For example, no changes were found in ERK activation in the Str after chronic cocaine administration
(analyzed 12 hr after the last injection) (Besson et al., 2000). In a CPP study, after 6 days of training, when animal is injected with saline (day 7) and re-exposed to the contextual cue chamber previously paired with cocaine exposure for 25 min, pERK activation was not found within the NAc (Besson et al., 2000). However, animals that received cocaine instead of saline and were re-exposed to the cocaine-paired chamber enhanced pERK. In this study, researchers were unable to detect any CPP induced-ERK activation unless the CPP test was accompanied with cocaine priming. During test session we exposed the animals just to the contextual cues previously paired with cocaine without any cocaine priming. It has been also seen that after 30 min of CPP test pERK is induced within the NAc core but not in the shell (Miller and Marshall, 2005). Thus, it is possible that in our experiment ERK activation within the NAc shell is not driving the expression of environmental elicited cocaine conditioning via mGluR5, reason of why no difference was obtained.

In addition, at one hour we were able to detect a reduction in the locomotor response when animals were pretreated with the mGluR5 antagonist, MPEP. As mentioned before, MPEP treatment reduced ERK phosphorylation levels (Mao et al., 2002; Schroeder et al., 2008). Even when MPEP diminished the behavioral response after one hour of contextual cues re-exposure, molecular changes in ERK activation mediated by MPEP treatment could be in an earlier time point of the conditioning test. Different studies have presented that DHPG, caused a rapid and transient increase in pERK1/2 levels (Yang et al., 2004; Topolnik et al., 2006), and in Fos expression (Park et al., 2010). This means that mGluRs response to effective pharmacological treatment is rapidly reflected in cellular protein modifications. Since MPEP presented a rapid effectiveness in mGluR5 blockade (Herzig and Schmidt, 2004), we speculate that in our conditioning protocol MPEP caused a rapid transient decrease in pERK levels after contextual cues and that’s why we were unable to detect the changes after one hour. Although these statements could explain why we did not detect any changes in ERK activation, we believe that within the cocaine-paired group the difference was not achieved because within vehicle treated animals, pERK was already dephosphorylated.
In conclusion, with these molecular studies, we were unable to detect any significant difference of Homer 1b/c and mGluR5 within NAc shell during any of the stages of environmental elicited-cocaine conditioning. These results demonstrate and support what was found by others (Mitrano et al., 2008), that even when cocaine evokes a glutamate imbalance (Smith et al., 1995; Pierce et al., 1996; Reid et al., 1997) the effects on trafficking of mGluR5 within the NAc shell is modest. However, there was a tendency of enhancement in these two proteins during acute exposure. It should be considered that synaptic localization of Homer is regulated within min by Ca\(^{2+}\) influx through NMDA receptors (Okabe et al., 2001) and that even in the presence of Homer, receptors are highly dynamic in their movements and in their composition (Serge et al., 2002). Homer 1b/c and mGluR5 modulated cocaine behavioral response, and it seems that their interactions are important in cue induced reinstatement of cocaine (Wang et al., 2013). Therefore, taking into account our previous behavioral and molecular results, we proposed that the inhibition of the expression of the conditioning, results in the disrupting of the intracellular signaling mediated by mGluR5, and it is proposed that the ability of Homers to mediate mGluR5 expression is unaffected. In addition, although we cannot have a clear response of which mGluR5 downstream elements are modulating the expression of the environmental elicited cocaine conditioning, we can conclude that after one hour of contextual cues re-exposure ERK activation is at its basal level of expression. We can suggest that mGluR5 within the NAc shell can induce the expression of environmental elicited cocaine conditioning through an early activation of ERK1/2. More experiments are needed in order to characterize the specific mechanism of action of mGluR5 in modulates the expression of the conditioning.
Chapter V

General Conclusions & Discussions
For the last decades cocaine has been one of the most common illicit drugs in our society (NIDA). Cocaine is a psychostimulant that causes addiction, an endurable disease that affects the brain and behavior that has major social impact. Addiction is characterized by a compulsive use of the drug despite of adverse consequence. Unfortunately, drug addiction is not viewed in our society as a disease, most likely, it is perceived as a moral weakness. While it is very probable that for an individual the first exposure to the drug was voluntary, the continuous use of the drug has long-lasting effects within the nervous system. All drugs of abuse including cocaine, tends to enhance DA levels within the reward pathway. Once it starts hijacking this neurocircuitry, the long term changes are revealed and addiction starts to develop. These long term changes within the brain can be prolonged for years even after long abstinence periods. Furthermore, when addicted people have been without access to the drug, withdrawal symptoms emerge as a negative affective state. It is believed that the avoidance of this negative affective state is what drives the person to the continuous search and use of the drug.

Chronic cocaine use lead to changes within mesocorticolimbic circuitry in brain regions that are involved in memory, learning, emotions, decision making among others. These changes within these brain regions after chronic cocaine use underlie addiction. Furthermore, it may be one of the main reasons why addiction is such a difficult disease to treat and eradicate. Even when addicts have cease drug use, for short or long periods, changes that have occurred in the reward neurocircuitry as consequence of drug abuse, leave the brain more vulnerable and as a result, we have an individual more susceptible to relapse into drug use.

Three major factors can evoke relapse after abstinent periods. One of these factors is the environment associated with previous drug use. It seems that during chronic cocaine use a strong association is established with the reinforcing properties of the drug and the environment around it. Thus, the subsequent exposure to these environmental cues can induce craving and desire to use the drug (Ehrman et al., 1992; Avants et al., 1995), as a consequence precipitate and trigger relapse
(Gawin, 1991). Even when addiction rehabilitation centers seem to be “effective” for some periods, most of the people after leaving the center, relapse into drug use. This clearly establishes and indicates that there it is not yet an effective treatment that can have endurable effects to eradicated addiction. Furthermore, this lack of effectiveness in the eradication of relapse can be translated into the fact that more research is still necessary in order to find a “cure” for addiction.

Because of the complexity of human studies, different protocols to investigate addiction have been developed with animals. Cocaine self administration and CPP are the most common protocols used to assess different behavioral and molecular responses evoked by re-exposure of animals to contextual cues previously paired with cocaine exposure. In addition, cocaine induced locomotion conditioning protocols have also been effective in demonstrating how re-exposure to environmental stimulus previously paired with cocaine administration can elicited a higher locomoting response when compared with saline animals. The present thesis contributes to the extensive evidence of the influence of environmental stimulus in the behavioral response to cocaine addiction using another approach. The learned associations that occur during the process of repeated drug use in addiction can elicit a different behavioral response when an encounter with a different stimulus occurs. The behavioral results obtained in our studies when animals were re-exposed just to contextual cues without any cocaine on board, models the effects that the addict feels when he/she encounters with environmental stimulus previously paired with drug use. Thus, animal models designed to understand the neurobiology of drug addiction maintains important relevance for the subsequent development of effective treatments and/or the cure for addiction.

Molecular studies of cocaine addiction have focused on the DA system as the principal neurotransmitter responsible for the rewarding effects of cocaine. It is a fact that VTA dopaminergic afferents to the NAc are of great importance in the development of addiction, but are not the only innervation affecting the development and expression of cocaine addiction. Glutamate is a major neurotransmitter within the brain that is strongly associated with the molecular substrates
that underlie cocaine addiction. Studies indicated that there are significant interactions between dopaminergic and glutamatergic inputs within the NAc (Sesack and Pickel, 1990, 1992). Moreover, it has been found that the re-exposure to contextual cues previously paired with cocaine exert an induction of glutamate release (Wilson et al., 2004; Kalivas, 2009; Koob and Volkow, 2010; for review, Gipson et al., 2013) within the NAc.

Glutamate is a major regulator of synaptic plasticity, learning and memory processes. It is known that cocaine influences glutamate transmission within the NAc, that in turn produces persistent changes in neuronal function which is translated into the behavioral effects of cocaine (For review, Schmidt and Pierce, 2010). It is suggested that glutamate neurotransmission is modulating the memory processes involved in cocaine addiction, including the ones associated with the environmental cues and cocaine rewarding properties. Behavioral results from the present thesis contributed to further characterize and confirm that mGluRs receptors, specifically mGluR5, modulates the expression of locomotion conditioning induced by re-exposure to contextual cues associated with the rewarding effects of cocaine. The specific mGluR5 mechanism driving the expression of cocaine conditioning is still unclear. Different mechanisms, including those that involve LTP and LTD may underlie the synaptic plasticity processes during cocaine addiction. It has been established that within the NAc shell those processes (LTP and LTD) occurred, and mGluR5 are involve in both of them. More experiments are needed to elucidate this assumption. In addition to LTP and LTD processes, mGluR5 cell localization within the NAc shell can be another focus of study to further characterize mGluR5 role during cocaine locomotion conditioning. There are gabaergic and cholinergic interneurons within the NAc subregions. Previous studies have shown that mGluR5 is expressed in dendrites of NAc cholinergic interneurons (Mitrano and Smith, 2007). Furthermore, Witten and colleagues (2010) demonstrated that cholinergic interneurons within the NAc regulate cocaine conditioning, since inhibition of these kinds of cells blocks the conditioning (Witten). Thus, it is possible that mGluR5 blockade within the NAc shell attenuates cholinergic
interneurons activity, resulting in a decrease of the conditioned locomotion. Future experiments can target cholinergic interneurons mGluR5 within the NAc shell to further characterize mGluR5 role within this brain subregion.

In addition to the behavioral experiments, we performed some molecular work. We obtained no major changes in the expression patterns of mGluR5 and Homer 1b/c proteins during the different stages of cocaine conditioning. Based on these findings, we propose that the inhibition of the expression of the conditioning, results in the disrupting of the intracellular signaling mediated by mGluR5, probably without affecting Homer 1b/c and mGluR5 expression during the acquisition and expression of the conditioning. In order to test this hypothesis, a tat-peptide that disrupts mGluR5-Homer1b/c interaction can be given to the rats and perform the behavioral conditioning. Although in the present study no differences in mGluR5 and Homer 1b/c was detected, different non-clinical and clinical studies report mGluR5 and Homer 1b/c protein level changes during cocaine addiction phases. Specifically, changes are more often during withdrawal and abstinence periods of cocaine exposition. For example, a clinical study showed that long-term cocaine use is associated with a decrease in mGluR5 availability compared with healthy control subjects (Martínez et al., 2014). Related with Homer 1b/c, increased levels were detected in cocaine users (Okvist et al. 2010). Thus, cocaine exposure affects mGluR5 and Homer 1b/c protein concentrations in human brain. These alterations may underlie synaptic plasticity in human drug abusers.

Biochemical evidence established that mGluR5 activation initiates an intracellular cascade that involves ERK1/2 activation. Activation of ERK1/2 leads to a signaling that involves expression of different transcription factors related to learning and memory processes. Given the fact that the expression of the environmentally elicited cocaine conditioning involves recall of associative memories, we hypothesized that expression of the conditioning will involve ERK1/2 activation within the NAc shell. In addition, we expected to see a reduction of pERK1/2 levels with an MPEP treatment. Results obtained do not allowed us to have a clear picture of what downstream effects
can account for the expression of the conditioning. After one hour of contextual cues re-exposure, cocaine-paired animals failed to show higher levels of pERK1/2. Moreover, MPEP did not affect the activation of ERK1/2 within the NAc shell. Even when the majority of the literature suggests that there is an induction of pERK within the NAc when animals are re-exposed to contextual cues previously paired with cocaine, some studies report no changes (Thomas et al., 2008). In addition, some of the studies that reported activation of pERK within the NAc are more directed to the core subregion and not the shell (Miller and Marshall, 2005). We can suggest that within the NAc shell ERK1/2 activation through mGluR5 is not one of the principal intracellular proteins driving the expression of environmental elicited cocaine conditioning. mGluR5 activation leads to different intracellular cascade signaling that eventually is translated into transcription factors activation and also in protein synthesis. Group I mGluRs, has been shown to activate the mammalian target of rapamycin complex1 (mTORC1) pathway which is implicated in neurobiological processes within the VTA and the NAc during addiction (Dayas et al., 2012). mTORC1 mediates behavioral signaling in long-term memory formation (Tischmeyer et al., 2003; Parsons et al., 2006a; Blundell et al., 2008) including the ones that involves expression of cocaine CPP (Dayas et al., 2012). In addition, mTOR activation alone is sufficient for synaptic protein translation and plasticity (Mameli et al., 2007; Ronesi and Huber, 2008). It has been demonstrated that protein synthesis is required for memory formation and consolidation (Nader et al., 2000; Schafe et al., 2000). Furthermore, the development of drug associated memories and addictive behavior also requires new protein synthesis (Mierzejewski et al., 2006a; Milekic et al., 2006; Kuo et al., 2007; Fei et al., 2013). It is known that mGluR5 activation, leads to an enhancement of protein synthesis at synapses (Weiler and Greenough, 1993). Thus, it is possible that re-exposure to environmental cues cause glutamate release which activates mGluR5 that in turn also activates mTORC1, increase protein synthesis and consequently modulate the expression of environmental elicited cocaine conditioning.
Although there is several reasons that can explain why we did not obtain any changes in pERK expression levels within the NAc shell we were able to detect a tendency of enhancement in the cocaine-paired animals. ERK has been implicated in synaptic plasticity (English and Sweatt, 1996; Martin et al., 1997) and in different process of learning (Atkins et al., 1998; Kelly et al., 2003) including those involving retrieval of memories for cocaine associated contextual cues (Mizoguchi et al., 2004; Miller and Marshall; 2005; Valjent et al., 2006). Therefore, we are more inclined to state that due to the time point selected for testing after re-exposure to contextual cues (previously paired with cocaine) we were unable to find significant differences in ERK1/2 activation within the NAc shell. ERK activation possibly dissipates by the time of tissue collection. This assumption suggests that ERK could be mediating a rapid effect in the expression of the conditioning. Supporting this assumption, it has been established by others that “ERK activity mediates the acute behavioral effects of cocaine cues over short time periods (min)” (Miller and Marshall, 2005; Lu et al., 2006). Future experiments can establish different time points after contextual cues re-exposure in order to clarify if mGluR5-pERK activation has a role during expression of conditioned locomotion. In addition, pharmacology studies to manipulate ERK phosphorylation can also be performed in order to asses ERK role during expression of the conditioning. We decided to measure pERK activation after 1 hour of re-exposure to environmental cues previously paired with cocaine, because at that time we were able to detect the highest behavioral response when animals are re-exposed to environmental cues.

With the present thesis we further characterize the role that mGluR5 have in modulating the incentive values given to a contextual cues previously associated with the rewarding effects of cocaine. Given the challenge that represents relapse phase in cocaine addiction disease, any study that contributes to a better understanding of what molecular proteins are driving relapse is of relevance for the development of effective treatments. Moreover, to target specific molecular proteins that are involved in contextual cues triggered by relapse are of particular interest in the
field of pharmacotherapeutics. Although more studies are necessary to further characterize the mGluR5 downstream mechanism within the NAc shell driving cocaine conditioning expression, we propose a mechanism. Based on the most recent data, mGluR5 could be modulating the cocaine behavioral response by different signaling mechanisms. Those mechanisms could involve ERK1/2, Protein Kinase C gamma (PKCγ) activation, protein synthesis, and mGluR5-Homer1b/c interactions (Dayas et al., 2012; Fei et al., 2013; Wang et al., 2013; Schmidt et al., 2013, 2014) (figure 1).

First, based in our findings and in previous experiments, we suggest a model that does not include changes in the expression of mGluR5 or Homer 1b/c within synaptosomal membrane fraction. We are bias to believe that intracellular cascade induced by coupling of these proteins with other scaffolding proteins within the NAc shell, can account for the expression of the conditioning. In addition, we still believe that this intracellular cascade will involve ERK1/2 phosphorylation within the NAc shell immediately after contextual cues re-exposure. ERK1/2 activation could be in part mediated by different mechanism such as PKCγ. In addition, ERK1/2 phosphorylation could lead to protein synthesis (involved in mediating synaptic plasticity such as PSD95, NMDAR subunits among others), and transcription factors activation such as CREB, and Elk-1 (Valjent et al., 2000; Brami-Cherrier et al., 2005; Page et al., 2006; for review, Brami-Cherrier et al., 2009; Bernard et al., 2011; and Dayas et al., 2012) that can be mediating the behavioral outcome. These are transcription factors that have a role in learning and memory processes, including the ones than involves cocaine addiction/exposure (Carlezon et al., 1998; Thomas, et al., 2008; Marin et al., 2009; Bernard et al., 2011). In response to extracellular stimuli, ERK is recruited to transfer signals from the cell membrane to downstream targets in the cytoplasm or nucleus, followed by immediate responses, long-lasting adaptive changes (For review, Zhai et al., 2008), or behavioral performance (Lee et al., 2005). With this assumption, we suggest that ERK activation could be mediating the first phase of the expression of the conditioning that eventually is translated into the behavioral response reflected in a later time period.
Figure 1. Possible intracellular mechanism of action of mGluR5 during environmental elicited cocaine conditioned locomotion.
Appendix I

Elucidating mGluR5 Intracellular Expression of PKC\(\gamma\) during Expression of Environmental Elicited Cocaine Conditioned Locomotion
Abstract

The NAc shell modulates the rewarding properties of cocaine. Within the NAc shell different mechanisms can be modulating these rewarding processes. Our results demonstrated that mGluR5 modulates the expression of environmental elicited cocaine conditioned locomotion. In our previous experiment ERK1/2 activation was assessed after the expression of conditioned locomotion in animals. PKCγ is an upstream element of ERK1/2, and it has been previously implicated in cocaine reward. The present experiment examines the PKCγ within the NAc shell in cocaine conditioned locomotion. Animals were implanted with cannulae within the NAc shell, and separate groups were exposed to the cocaine conditioned locomotion paradigm. In the test session (D12), separate groups of animals were infused within the NAc shell with 25nmol/0.5µl/side of MPEP. Animals were sacrificed and immunoblots for the NAc shell subregion were performed. Results show no changes for PKCγ within the NAc shell after the blockade of mGluR5 receptors.

Introduction

PKC phosphorylation levels of some of its isoforms increased within the NAc with cocaine exposure (Steketee et al., 1998). In addition, PKC have been previously implicating in regulating psychostimulants behaviors. For example, CPP behaviors are attenuated with systemic inhibition of PKC (Cervo et al., 1991). Moreover, cocaine sensitization is blocked with microinjection of a PKC inhibitor directly into the NAc (Pierce et al., 1998), and cocaine reinstatement is blocked by PKC inhibition (Schmidt et al., 2014).

Group I mGluRs activates PKC (Conn and Pin 1997), and recent data demonstrated that cocaine priming-induced reinstatement is mediated by mGluR1/5, and PKCγ activation within the NAc core (Schmidt et al., 2014). Consistent with this data Schmidt et al. 2013 recently demonstrated that cocaine reinstatement correlated with an increase activation of PKCγ, but not PKCα or PKCβII, within the NAc shell (Schmidt et al., 2013). Furthermore, PKC inhibition within the NAc shell was enough to attenuate DHPG induced cocaine seeking reinstatement (Schmidt et al., 2014).
Since cocaine reinstatement correlates with an increased activation of PKCγ within the NAc shell, and mGluR group I elicited PKC activation, it is proposed that mGluR5 blockade within the NAc shell attenuates environmental elicited-locomotion conditioning via a decrease of PKCγ activation.

**Material and Methods**

***The PKCγ immunoblots were performed using the same homogenized tissue that was used to performed ERK1/2, and pERK1/2 immunoblots. For the complete procedure used, please refer to chapter 4.

**Immunobloting**

Western blot analyses for PKCγ were performed as previously described (refer to Chapter 4, material and methods section) with the primary antibody against PKCγ (1/1000) (Santa Cruz Biotechnology, sc-211) over night at 4°C.

**Results**

**mGluR5 blockade within the NAc shell does not attenuate expression of PKCγ phosphorylation**

We decided to investigate if mGluR5 blockade within the NAc shell disrupts the expression of environmental elicited cocaine conditioned locomotion via PKCγ. Results showed that PKCγ expression was unaffected during the expression session (Figure 1). In addition, MPEP treatment was ineffective in altering PKCγ protein levels within any of the groups.

**Discussion**

Before knowing the results of pERK1/2 protein levels after expression of the conditioning, we were expecting an enhancement of PKCγ activation with contextual cues re-exposure in the cocaine-paired animals. In addition, we were also expecting MPEP to attenuate this inducement of
pPKCγ activity. Results of the pERK1/2 levels showed no changes within the NAc shell after re-exposure to environmental cues previously paired with cocaine. Therefore, we expected not to detect any changes in PKCγ levels. However, it could be possible that phosphorylation levels of PKCγ were varying within the different groups (phosphorylation levels were not measured).

Based on the literature and not in our previous results of pERK1/2, we are expecting to detect different protein patterns of expression of pPKCγ within different groups. First, we were expecting no difference of PKCγ levels within cocaine-paired vehicle treated animals, however we are also expecting to detect an increase of pPKCγ during the first min following the contextual cues re-exposure. This increase would correlate with the higher locomotor response of cocaine-paired animals with contextual cues re-exposure. In addition, we were expecting to detect decrease in PKCγ within cocaine-paired animals with MPEP treatment. Supporting this notion the study of Schmidt et al., (2013). They report that the reinstatement of cocaine seeking correlated with an increased expression of PKCγ activation (Schmidt et al., 2014). However, the behavioral response was reported at 120 min of cocaine induced reinstatement, but the immunoblots for PKCγ activation was performed after 30 min of the behavioral procedure.
Figure 1. PKCγ protein levels within the NAc shell during the expression of environmental elicited cocaine conditioned locomotion. A. Representative immunoblots of PKCγ and its loading control GAPDH within the NAc shell during expression session, N=Naïve, CV=Control Vehicle, CM=Control MPEP, PV= Cocaine-paired vehicle, PM= Cocaine-paired MPEP, UV= Cocaine-Unpaired Vehicle and, UM= Cocaine-Unpaired MPEP. B. Representative graphs for PKCγ. A pool of 4 animals per group was submitted to immunoblots test. Two different pools were assessed and the overall results are presented in the graphs (a total of 8 animals per each graph bar).
PKCγ expression within the NAc shell during expression of environment-elicited cocaine-conditioning

A.

PKCγ

GAPDH

N  CV  CM  PV  PM  UV  UM

B.

PKCγ expression within the NAc shell during expression session

Vehicle  |  MPEP

PKCγ/GAPDH

Naive  |  Control  |  Cocaine-Paired  |  Cocaine-Unpaired

0.0  |  0.1  |  0.2  |  0.3  |  0.4  |  0.5
Group I mGluRs within the NAc core modulates environment-elicited cocaine conditioning expression.
Abstract

We previously demonstrated that mGuR5 within the NAc shell affected the expression of environmental elicited cocaine conditioning. During day 12 of our locomotion conditioning protocol, a recall process of associative learning from the contextual cues previously paired with cocaine rewarding effects was assessed. Several studies have established that both NAc subregions are involved in the expression of contextual cues induced cocaine reinstatement, CPP, sensitization or locomotion conditioning. Most of the literature reported that the NAc core is a region within the NAc that is involved in eliciting the cue induced reinstatement and CPP. In this experiment we want to determine if group I mGluRs within the NAc core elicit cocaine conditioned locomotion. For this experiment, animals were implanted with cannulae within the NAc core, and separate groups were exposed to our conditioned protocol. In the test session (D12), animals were exposed to the multimodal environment without any cocaine or saline pre-treatment. Before placing the rats in the chambers, separate groups of animals were infused within the NAc core with 10nmol of CPCCOEt, an antagonist of mGluR1, 25nmol of MPEP, an antagonist of mGluR5, or with vehicle. Blockade of the mGluR1 and mGluR5 subtype decreased conditioned locomotion in the cocaine-paired groups. Here we demonstrated that both group I mGluRs subtypes within the NAc core are involved in the expression of environmental elicited cocaine conditioning. Our results suggest that group I mGluRs within the NAc core positively modulates the expression of memory related to the association of environmental cues with the rewarding effects of cocaine. In addition we found NAc core specificity for mGluR1 modulation in the expression of environmental elicited cocaine conditioning. Moreover, mGluR5 modulate the expression of the conditioning within both subregions.
Introduction

The neurobiology effects of cocaine affect a whole neurocircuitry that involves different brain regions. Within these brain regions the NAc plays a crucial role in the rewarding effects of cocaine addiction (Everitt et al., 1999; Ikemoto and Panksepp, 1999; Parkinson et al., 2000). Based in its inputs, this brain region have been established to be a site of integration of emotional salience, specific contextual information, and executive motor plans, which is translated into goal-directed behavior including the ones related to drug addiction (For review, Goto and Grace, 2008; Wolf, 2010) In addition, the NAc is critical for the expression of Pavlovian stimulus-reward relationships (Day and Carelli, 2007).

The NAc is subdivided in two sub-regions, the shell and the core. Based on their connectivity, the NAc shell has been defined as part of the limbic system, while the NAc core is associated more to the dorsal Str and basal ganglia (Alheid and Heimer, 1988). Both sub-regions have a role in mediating the association of contextual cues and cocaine rewarding effects. However, the literature attributed a more specific role of the NAc core in regulating the incentive value of reward to conditioned stimuli (Di Ciano and Everitt, 2004; Fuchs et al., 2004; Ito et al., 2004; Saunders et al., 2013).

Glutamate neurotransmission within the NAc is modulated by cocaine exposure, and an extensive body of evidence centers it as a key regulator of the neuroplasticity that underlies addiction. Glutamate, the major excitatory neurotransmitter in the brain, exerts its action in the NAc through both ionotropic and mGluRs. mGluRs are divided in three different groups based in their homology and second messenger intracellular cascade (Conn and Pin, 1997). Group I mGluRs includes mGluR1 and mGluR5 subtypes, they are coupled to a Gq protein and activate PLC intracellular cascade which results in DAG and IP3 generation (Kim et al., 2008; Conn and Pin, 1997). Group I mGluR subtypes are found within both NAc subregions and it seems that their
action mediate some of the neuroadaptive changes associated with cocaine addiction (Kenny and Markou, 2004; Mitrano et al., 2008).

Substantial evidence indicated that group I mGluRs receptors, modulate cocaine-mediated behaviors. For example, genetic deletion of mGluR5 abolished cocaine self administration (Chiamulera et al., 2001). Additionally, different pharmacology studies using an mGluR5 antagonist attenuated expression of cocaine sensitization, CPP, contextual cues induced locomotion conditioning and, also attenuates the reinforcing effects of cocaine (McGeehan et al., 2003; Tessari et al., 2004; Herzig et al., 2005; Kenny et al., 2005; Lee et al., 2005; Paterson and Markou, 2005; Platt et al., 2008; Kim et al., 2008; Kumaresan et al., 2009; Martinez-Rivera et al., 2013). In relation to mGluR1, results are less available but there is evidence that mGluR1 also modulates behavioral response related with psychostimulants addiction. For example, mGluR1 antagonist reduced behavioral sensitization to chronic cocaine administration in rats (Dravolina et al., 2006). Related to reinstatements studies, it was shown that blockade of mGluR1 attenuated cue-induced cocaine reinstatement (Xie et al., 2010, 2012; Achat-Mendes et al., 2012).

Previous results from our laboratory found a role for mGluR5 within the NAc shell in the expression of environmental elicited cocaine conditioning (Martinez-Rivera et al., 2013) but not for mGluR1. To further characterize group I mGluRs role in the expression of the behavioral effects of cocaine, we decided to block mGluR1 and mGluR5 (independently) within the NAc core during expression session of locomotion conditioning. Results demonstrated that blockade of both receptors attenuated the locomotion response with re-exposure to contextual cues previously paired with cocaine. These results suggests that activation of group I receptors are necessary for recall of memories related with the associative learning of contextual cues and previous cocaine rewarding effects.
Material & Methods

Subjects: Male Sprague Dawley Rats (250-275 g) purchased from Charles Rivers Laboratories (Wilmington, MA).

Cannulae implantation surgery and microinfusions: Surgery was performed as previously described (chapter II and III). The 10mm guide cannulae were implanted using a stereotaxic procedure within the NAc core (A-P +3.5, M-L ±2.0, D-V -5.3) and rats were placed with the nose bar 5.0 mm above interaural zero. For intracerebral microinfusions we used 12.5 mm injector cannulae. Each rat received only one concentration of MPEP (25nmol) (Martínez-Rivera, et.al 2013), CPCCOEt (10nmol) (Swanson & Kalivas, 2000), or vehicle. At the end of the session, the animals were sacrificed, their brains removed and frozen for histology analysis. For details, refer to materials and methods section in chapter II and III.

Conditioning methods. Animals were subjected to one of three different groups: cocaine-paired (experimental), controls, and cocaine-unpaired. Conditioning details was previously described in chapter II and III.

Statistical Analysis; The behavioral parameter used to analyze our data was AD. For the test session, data was analyzed using a two-way ANOVA to determine whether drug conditioning depends on the antagonist used and whether there is a possible interaction of antagonist microinjection with the conditioning. Post hoc multiple comparison analyses were performed using Tukey test when significance were obtained.

Results

Histological analysis

Placement of the cannulae within the NAc core was verified using cresyl violet staining. Figure 1 shows a schematic representation of rat brains sections with the approximate localization
of the cannulae placement. Animals with cannulae outside of the area of study were excluded from analysis. Most of the cannulae were positioned between + 1.70 and + 1.20 from bregma. A total of 87 animals were found to have their cannulae within the NAc core.

**Effects of Group I mGlurs blockade within the NAc core**

In order to assess the role of mGluR1 during the expression session (D12), animals were microinjected with CPCCOEt within the NAc core and returned to the activity chambers with the environmental cues but without cocaine or saline injections for a 90 min period (figure 2a). Statistical analysis of the AD parameter revealed that the expression of the conditioning depends on CPCCOEt treatment \( F(2, 45) = 5.548, p<0.05 \). Moreover, there is a statistically significant interaction between conditioning and CPCCOEt treatment \( F(2, 45) = 3.991, p<0.05 \).

A post hoc analysis demonstrated that comparisons for the conditioning factor within the vehicle showed that the cocaine-paired group \((n=9)\) presented a greater AD over control \((n=8, p<0.05)\) and cocaine-unpaired animals \((n=7, p <0.01)\), indicating that environment-elicited cocaine conditioning was present. No difference was obtained within control and cocaine-unpaired animals. In addition CPCCOEt attenuated locomotor AD response within cocaine-paired subjects (cocaine-paired vehicle \((n=9)\) vs. cocaine-paired CPCCOet treated animals \((n=6)\) p <0.01) but not within control or cocaine-unpaired animals.

In addition, the same procedure was repeated with another set of animals microinjected within the NAc core with MPEP an mGluR5 subtype antagonist. A two way ANOVA analyses presented a significant conditioning effect \( F(2, 52) = 4.224, p<0.05 \). A post hoc analyses
Figure 1. Schematic representations of cannulae placement within the Nac core. Circles symbols represent the approximate bilateral cannulae location within the NAc core. Animals were microinjected with CPCCOEt, MPEP or vehicle. A total of 87 animals were used. Injector sites may appear fewer than the reported number of rats because of overlap of placements.
Fig. 1

Schematic Representation of the cannulae placements within the NAc core
demonstrated that re-exposure to environmental cues, previously paired with cocaine elicited a higher locomotor response. Within vehicle treated animals, cocaine-paired subjects \((n=9)\) presented a greater AD over control \((n=8, \ p<0.05)\) and cocaine-unpaired animals \((n=7, \ p<0.01)\). This response was attenuated with MPEP treatment within cocaine-paired animals (cocaine-paired vehicle \((n=9)\) vs. cocaine-paired MPEP treated animals \((n=10)\) \(p<0.01\)) but not within control or cocaine-unpaired groups.

**Discussion**

Expression of environmental elicited cocaine locomotion conditioning involves a process of association between the contextual cues and the previous rewarding effects of cocaine. Recall of those memories may explain why the animals present a higher locomotor response when re-exposed to contextual cues. With the present experiment, we were able to replicate our previous behavioral studies related to the NAc shell subregion. Results obtained in this experiment showed that animals re-exposed to contextual cues previously paired with cocaine injections presented a higher locomotor response. In addition, blockade of mGluR1 with CPCCOEt, and blockade of mGluR5 with MPEP within the NAc core attenuated the response elicited by contextual cues within cocaine-paired animals.

Our results further confirmed previous data that reported a role of the NAc core in strengthening the association of contextual cues and cocaine behavioral response (Di Ciano and Everitt, 2004; Fuchs et al., 2004; Ito et al., 2004; Saunders et al., 2013; Wang, 2013). This significant reduction is cocaine reward related, since blockade of any mGluRs group I subtypes does not attenuate locomotion response behaviors in the control or the cocaine-unpaired subjects. This could imply that group I mGluRs receptors are modulating the memory processes regulated within the NAc core for the association of the environmental cues with cocaine effects.
Figure 2. Effects of mGluR1, and mGluR5 blockade within the NAc core on the drug expression session. The parameter used to measure response was the AD in 90 min (measurements are in seconds. 2a. Cocaine-paired vehicle subjects presented a higher locomotor activity than control vehicle subjects (*p<0.05) and cocaine-unpaired vehicle subjects (**p<0.01). Blockade of mGluR1 with 10nmol of CPCCOEt significantly decreased the locomotor activity of the cocaine-paired animals (cocaine-paired vehicle vs. cocaine-paired MPEP treated animals (§§ p< 0.01) no difference was obtained between control subjects neither between unpaired subjects. 2b. For the mGluR5 studies, cocaine paired vehicle subjects presented a higher locomotor response than control (* p<0.05) and cocaine-unpaired vehicle subjects (**p<0.01). Blockade of mGluR5 with 25nmol of MPEP significantly decreased the locomotor activity of the cocaine-paired animals (cocaine-paired vehicle vs. cocaine-paired MPEP treated animals (§§ p< 0.01) no difference was obtained between control subjects neither between unpaired subjects.
Fig. 2

**mGluR1 blockade within the NAc core during expression session**

(A) 

- Y-axis: Ambulatory Distance (cm/90 min)
- X-axis: Control, Cocaine-Paired, Cocaine-Unpaired
- Graph showing comparison between Vehicle and CPCCOet

(B) 

- Y-axis: Ambulatory Distance (cm/90 min)
- X-axis: Control, Cocaine-Paired, Cocaine-Unpaired
- Graph showing comparison between Vehicle and MPEP
Previous studies have demonstrated that DHPG, induced an increase of extracellular glutamate levels (Herrero et al., 1992; Moroni et al., 1998; Reid et al., 1999; Swanson et al., 2001), meaning that mGluR1/5 activation induced glutamate release. In addition, the presence of contextual cues previously paired with cocaine intake can evoke craving and drug seeking behavior that is related with an activation of the PFC glutamatergic afferent of NAc (Moroni et al., 1998; Wilson et al., 2004; Kalivas, 2009; Koob and Volkow, 2009). It is very probable that in our experiment, contextual cues re-exposure activated PFC glutamatergic projection eliciting glutamate release into the NAc core. Eventually, mGluR1/5 were activated which in turn promoted the subsequent glutamate release that contributed to the higher behavioral response. We demonstrated that mGluR1/5 blockade within the NAc disrupted expression of environmental elicited cocaine conditioning. Blockade of these receptors disrupted the association of contextual cues previously paired with cocaine probably, by promoting a reduction of glutamate release which have been substantially proven to mediate memory processes. Additionally, disrupting the mGluR1/5 intracellular cascade activation inhibits an intracellular signaling that modulates the associative learning involved in detecting cues previously paired with cocaine (Cleva et al., 2010).

Different actions of mGluR5 within the NAc core can be involved in the modulation of the cocaine related behaviors reported in this study. As previously proposed (Martinez-Rivera et al., 2013) the reduced expression of the conditioning could be mediated by the lack of modulation of NMDAR by mGluRs group I activation. mGluR are positively coupled to NMDAR and it has been suggested that mGluR5 modulates NMDA Long Term Potentiation (LTP) (Francesconi et al., 2004; Grueter et al., 2007; Jia et al., 1998; Lee et al., 2002). It is possible that this mGluR blockade results in NMDAR plasticity modification.

Another mechanism within the NAc core that may be mediating mGluR5 environment-elicited cocaine conditioning can be through Homer proteins and intracellular cascade activation. Recently, it was demonstrated that disruption of mGluR-Homer1b/c interactions disrupted cocaine
cue induces reinstatement (Wang, 2013). Thus, it is possible that group I blockade affected the intracellular cascade associated with Homer1b/c protein which has been proven to mediate cue induced reinstatement.

In conclusion, we were able to further characterize group I mGluR role during expression of environmental elicited cocaine conditioning. We demonstrated that activation of both receptors independently within the NAc core is necessary for expression of locomotion conditioning. Future studies are needed to characterize the specific mechanisms of action of mGluR1 and mGluR5 involved in the expression of environmental-elicited cocaine conditioning.
Appendix III

Analyses of the AD during expression of environmental-elicited cocaine conditioning of animals with cannulae out of the NAc shell subregion
This section includes all rats that were eliminated for the results and analyses performed in chapter II. These animals were eliminated because the cannulae were out of the NAc shell subregion. Statistical Analyses for these animals was not performed because in some of the groups there is only one animal.

Table 1. Animal's microinjected with mGluR1 vehicle or antagonist

<table>
<thead>
<tr>
<th>Rat ID</th>
<th>Conditioning</th>
<th>Antagonist or vehicle</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OG 4</td>
<td>Control</td>
<td>Vehicle</td>
<td>1608.836</td>
</tr>
<tr>
<td>OG 6</td>
<td>Control</td>
<td>Vehicle</td>
<td>1537.208</td>
</tr>
<tr>
<td>OG 7</td>
<td>Control</td>
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<tr>
<td>QF 25</td>
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<tr>
<td>QI 11</td>
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<td>OM 21</td>
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<td>OK 8</td>
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Animals microinjected with mGluR1 antagonist

![Graph showing Ambulatory Distance (cm/90 min) for different conditions and treatments](image)
Table 2. Animal’s microinjected with mGluR5 vehicle or antagonist

<table>
<thead>
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<th>AD</th>
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<td>PG 4</td>
<td>Cocaine-Paired</td>
<td>MPEP</td>
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</table>

Animals microinjected with mGluR5 antagonist

![Ambulatory Distance Chart]

- **Vehicle**
- **MPEP**
Table 3. Animal’s microinjected with Group II mGluR vehicle or antagonist

<table>
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Table 4. Animal’s microinjected with Group III mGluR vehicle or antagonist

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<td>CPPG</td>
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