Multiple Cell Signaling Pathways Modulate the Cocaine-Induced Increase in Mu Opioid Receptor Protein Expression in PC12 Cells

by

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ABSTRACT

MULTIPLE CELL SIGNALING PATHWAYS MODULATE THE COCAINE-INDUCED INCREASE IN MU OPIOID RECEPTOR PROTEIN EXPRESSION IN PC12 CELLS

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University of Guelph, 2013

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Cocaine is interrelated with the opioid system on many levels, especially via the mu opioid receptor (MOR). Also, cocaine has been involved in modulating nitric oxide (NO) actions within the cell. The effect of cocaine was first assessed on the MOR, and then on transcription by the use of 1 µg/mL actinomycin D inhibitor. Several signaling pathways that cocaine may exert its action in modulating the MOR up-regulation in protein expression were also explored. Two dosage regimens were used in cocaine treatment, single continuous treatment (SCT), and repeated intermittent treatment (RIT). Different pathway inhibitors were used on PC12 cells, as follows: the PLC-PKC inhibitors 5 µM U-73122 and 10 µM BIS-1 used to investigate the involvement of the PKC signaling pathways in MOR expression levels, the evaluation of MAPK pathway by the use of 50 µM U0126 inhibitor, and the 10 µM LY94002 inhibitor was used to investigate the PI3K/Akt pathway. Moreover, the effect of NO on these signaling pathways was investigated by the use of 20 mM nonselective L-NAME inhibitor and qualitatively by DAF-2 florescence. Western blot analysis indicated that cocaine up-regulated MOR protein expression. Also, RIT cocaine treatment increased MOR protein levels via transcription. All three signaling pathways, MAPK, Akt and PKC modulated cocaine-induced increase of MOR following SCT cocaine treatment (post-transcriptional). Both MAPK and Akt have
been found to modulate the cocaine-induced transcription of MOR via the two dosage regimens of cocaine, *SCT* and *RIT*. Also, inhibition of both PLC and PKC did not prevent cocaine-induced increase in MOR transcription, according to *RIT* of cocaine. Furthermore, Akt and PKC appeared to modulate cocaine-induced NO production while MAPK did not. NO seemed to be involved with the PKC and Akt pathways in up-regulating MOR in *RIT* of cocaine directly by the Akt pathway, and indirectly by the PKC pathway. On the other hand, NO and MAPK modulated the MOR up-regulation expression simultaneously, but in an individual/parallel manner. Furthermore, signaling pathway activation levels were tested using L-NAME which concluded that NO modulated cocaine-induced increase in total Akt protein levels, but did not appear to have an effect on phosphorylated MAPK activation levels. In conclusion, different treatment regimens of cocaine activate different pathways; *SCT* of cocaine activated all three signaling pathways, however, *RIT* of cocaine activated only the MAPK and Akt pathways.

Keywords: cocaine, mu opioid receptor, PKC, MAPK, Akt, PC12 cells, nitric oxide.
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DECLARATION OF WORK PERFORMED

I declare that all work reported in this thesis was performed by me.
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<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAF-2</td>
<td>4, 5-diaminofluorescein diacetate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle media</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalogram</td>
</tr>
<tr>
<td>EOP</td>
<td>endogenous opioid peptides</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N\textsuperscript{\textomega}-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen–activated protein kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>modified Eagle media – removed from methods</td>
</tr>
<tr>
<td>MOR</td>
<td>mu opioid receptor</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<td>Abbreviation</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>RIT</td>
<td>repetitive intermittent treatment</td>
</tr>
<tr>
<td>SCT</td>
<td>single continuous treatment</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline, 0.1% Tween20</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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INTRODUCTION AND REVIEW OF THE LITERATURE

A major goal of research on drug addiction is to develop effective treatments to deal with the long term behavioral disorders especially relapse induced by the addictive drugs such as cocaine. Cocaine addiction is a chronic relapsing disease in which cocaine's administration becomes the primary stimulus that drives behavior regardless of the adverse consequence that may ensue (Stewart 2003). As cocaine use becomes more compulsive, motivation for natural rewards that normally drive behavior decreases (Stewart 2003). The discontinuation of its use is associated with somatic signs of withdrawal, dysphoria, anxiety, and anhedonia (Gipson et al., 2013; Kalivas et al., 2012). Given that relapse can occur even after a long period of abstinence, it is hypothesized that such a maladaptive behavior is due to persistent neuroplastic changes in the brain induced by chronic cocaine abuse (Shippenberg et al., 2007). In fact, even after prolonged periods of abstinence, 80-90% of substance dependent individuals relapse (Sun et al., 2009). The high rate of relapse to cocaine-seeking behavior has become a trademark of cocaine addiction.

Human patients vary widely in their responses to addiction therapy and that situation generated a search for markers of treatment response (Ciraulo et al., 2003). A variety of psychosocial and cocaine use variables have been found predictive of treatment response at a group level in small scale studies, including duration of cocaine use, severity of addiction, employment status, and education level (Ciraulo et al., 2003; Hser et al., 2001; Kampman et al., 2002; McKay et al., 1999; McMahon et al., 2001; Poling et al., 2007; Reiber et al., 2002). Yet, there is no proof of any treatment to cocaine addiction.
According to Health Canada in 2011 among youth aged from 15 to 24 years, cocaine use decreased from 11.3% in 2004 to 4.8%. Although the rate of use declined, studies show that 75% of all cocaine users become addicted to the drug, and without help from cocaine addiction treatment or rehab, only 1 in 4 people quit cocaine on their own.

Another major goal of addiction research is the identification of the neural mechanisms by which drugs of abuse produce these effects. The molecular mechanisms underlying substance-related disorders still remain unclear. Also, the search for biological prognostic markers had limited success for cocaine relapse (Elkashef and Vocci 2003). On the other hand, studies showed electroencephalogram (EEG) patterns (Prichep et al., 1999), and peripheral measures of dopamine and serotonin function (Patkar et al., 2003; Patker et al., 2004) have been predictive of relapse by cocaine addicts in treatment in small size studies, but these findings have not been replicated with larger sample sizes or by other research groups. Insight into the cellular events modulated by cocaine will contribute to our understanding of the complex and multi-factorial neurobiology of cocaine addiction.

The opioid system and Mu opioid receptor (MOR)

Opioid receptors are distributed throughout the brain and spinal cord and are known to mediate a number of activities including analgesia, species-typical behavior, and reward. Both endogenous opioids, which are naturally produced within the body, and exogenous opiates, which are produced outside the body, produce a variety of symptoms including pain relief, euphoria, respiratory depression (rarely clinically harmful), constipation, nausea, and vomiting. The effects are produced by opioids
binding to opioid receptors throughout the body. Pharmacologists and molecular biologists have demonstrated that opioids act at three distinct classes of receptors: kappa, delta, and mu, although it is likely that additional subtypes exist (review in Dhawan et al., 1996). Since each class of receptor has a unique effect on the cell, the multitude of classes allows opioids to have a wide range of effects in the body.

Opioid receptors have a common general structure. Cloning demonstrates that the receptors are usually G protein-linked receptors imbedded in the plasma membrane of neurons (Satoh and Minami, 1995). Once the receptors are bound, a portion of the G protein is activated, which allows it to diffuse within the plasma membrane. The G protein moves within the membrane until it reaches its target, which is either an enzyme or an ion channel. Most often, the targets alter protein phosphorylation and/or gene transcription, which alter the short-term and long-term activity of the neuron, respectively. Although opioids usually activate G proteins, it was demonstrated previously that opioids occasionally act independently of G proteins. A key study found that DAMGO, a selective mu receptor agonist, modulates calcium-dependent potassium channels independently of G proteins in bovine adrenal medullary chromaffin cells (Twitchell and Rane, 1994). The finding further highlights the complexity of the opioid system.

Mu opioid receptors (MORs) are present both presynaptically and postsynaptically in neurons (review in Simon, 1991). When acting at presynaptic receptors, the peptides function as neuromodulators affecting the release of neurotransmitters. At postsynaptic receptors, the peptides act as neurotransmitters by directly altering membrane potentials. The overall effect of opioids on a particular
tissue depends upon the concentration and location of particular opioid receptors in the area.

The opioid system is connected with most neurotransmitter networks in the body. The interaction between the opioids and the dopaminergic system appears to be involved in addiction, tolerance, and withdrawal symptoms (see below). The relevant interaction appears to occur along the mesolimbic projection, particularly in the ventral tegmental area (VTA) and nucleus accumbens (NAc).

**The role of dopamine in cocaine addiction**

The mesolimbic dopaminergic system is the main system/pathway involved in the experience of pleasure, shown in Figure 1. Cocaine acts on the mesolimbic DA pathway of the midbrain, which extends from the brains prefrontal cortex (PFC), the VTA to the NAc. These areas control the reward (motivation), pleasure (euphoria) feelings, compulsion, and preservation (Everitt et al., 2005; Volkow et al., 2010; Wise et al., 2008). In addition, the limbic system has many structures related to the reward pathway which is a set of brain structures including the hippocampus, amygdala, anterior thalamic nuclei, septum, limbic cortex and fornix and also operates by influencing the endocrine system and the autonomic nervous system. However, the NAc which is considered the brain's pleasure center, is the most influenced brain structure in cocaine abuse.
Figure 1: The mesolimbic dopamine system. It includes the ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex (PFc). (www.scientificamerican.com)

Although cocaine has a variety of pharmacological actions, it is thought that the reinforcing properties of cocaine are mediated by its ability to inhibit the reuptake of dopamine (Anderson et al., 2008; Conrad et al., 2010). Studies hypothesized that cocaine binds to the dopamine transporter and prevents the reuptake of dopamine into presynaptic terminals, thereby increasing the concentration of dopamine in the synapse. Much of what we have learned about the role of DA in addiction has come from animal models of drug self-administration. Studies on rats indicated that DA receptors are effected significantly in the NAc and the VTA areas since drugs of abuse have specific molecular structures that in either direct or indirect ways act on the neuronal mechanisms that can produce reinforcing effects (Shippenberg et al., 2007; Schmidt et al., 2006; Everitt et al., 2005). Dopamine receptor antagonists blocked the cocaine-induced reward pathway (Berglind et al., 2006; Conrad et al., 2010). These studies also indicated that it appears that the shell of the NAc plays a role in the initial rewarding effects (Ikemoto, 2003), while the core is important for drug seeking (Fuchs et al., 2004). In
conclusion, the activation of the mesolimbic DA system has been related to cocaine addiction in animal models (Everitt et al., 2005).

### The role of the opioid and dopaminergic systems in cocaine addiction

At this time there is no broadly effective treatment for cocaine addiction in humans (Karila et al., 2012; Knapp et al., 2007; Gorelick 2005; Simpson et al., 2002). Over the last two decades, increasing evidence has implicated the opioid system in playing a role in cocaine addiction (see below). A better understanding of the role of this system may identify novel therapeutic targets. It was reported that opiate receptor antagonists can attenuate the rewarding effects of cocaine (Reid et al., 1993; Corrigall et al., 1991), also suggesting an interaction between central opioid and dopaminergic systems in cocaine reinforcement. Moreover, a key study indicated that chronic exposure to cocaine has been shown to alter MOR densities in specific regions of rat brain (Cohen et al., 1991; Unterwald et al., 2001). Since the regions where upregulation occurred are rich in dopaminergic neurons, it pointed out the possibility that dopaminergic activity can also influence the expression of the MOR and suggested that the endogenous opioid system, MOR in particular may play an important role in mediating some aspects of cocaine reinforcement and addiction (Azaryan et al., 1996; Unterwald et al., 2001). Also, studies indicated that cocaine, acting via D2 dopamine receptors, can cause the release of an endogenous opioid peptide (EOP) that binds to MOR within the NAc. Thus, up-regulating the MOR (Avery et al., 2009). Other studies demonstrated an increase in β-endorphin release in the NAc following experimenter-delivered and self-administered cocaine mediated by the local dopaminergic system. These findings also suggested that activation of the β-endorphin neurons within the NAc may be important in the
neurobiological mechanisms underlying the behavioral effects of cocaine (Roth-Deri et al., 2003). Moreover, in the investigation of the role of MORs in the reinforcing effects of cocaine use, a study investigated cocaine-induced conditioned place preference in MOR-deficient mice and their wild-type counter-parts were tested for comparison. Then, dopamine D1 and D2 receptor binding was measured. Results showed that there was no difference in dopamine D1 receptor binding, whereas dopamine D2 receptor binding was significantly lower in the hippocampus of deficient animals. This also reinforced the interaction between the opioid system and the dopaminergic systems in response to cocaine (Becker et al., 2002). Furthermore, the signaling pathways, that will be studied later on, were associated with dopamine receptors that have been proposed to play a critical role in drug-induced neuroadaptations in the brain (Cohen et al., 1991; Becker et al., 2002).

**Cocaine and the mu opioid receptor in rats**

In order to emphasize additionally on the subject, many studies investigated cocaine addiction using rat models (Sun et al., 2013; Chen et al., 2013; Purgianto et al., 2013). Chronic cocaine exposure was shown to affect the expression of opioid receptors. The increase in MOR density specifically, has been reported in brain regions after steady dose 14-day ‘‘binge’’ pattern in the NAc, the caudate putamen, and the cingulate cortex (Collins et al., 2002; Unterwald et al., 1993). Moreover, systemic administration of opioid antagonists consistently decreased cocaine-induced conditioned place preference (Gerrits et al., 1995; Houdi et al., 1989; Kuzmin et al., 1997), depressed cocaine self-administration, and delayed cocaine initiation on the following day (Lesscher et al., 2005; Kiyatkin and Brown, 2003). Recent studies also indicated that administration of MOR antagonist, decreased cocaine seeking behavior.
in a dose-dependent manner. Thus, suggested that MOR antagonist may have therapeutic potential to reduce the tendency to seek cocaine and, additionally, to diminish the consequence of an initial relapse (Giuliano et al., 2013).

Although there are several studies in rat models showing that the opioid system plays an important role in cocaine addiction (Wee et al., 2010; Cummins et al., 2009; Roth-Deri et al., 2008; Shippenberg et al., 2007), fewer studies have examined the role of the opioid system in cocaine withdrawal. Thus, in order to determine whether cocaine and/or chronic withdrawal from cocaine alters the specific components of the opioid system, a study was carried out investigating MOR binding in the brains of rats treated with an escalating dose "binge" cocaine administration paradigm, and of rats chronically withdrawn from cocaine (Bailey et al., 2005). Male Fischer rats were injected with saline or cocaine at 1h intervals for 14 days. As shown in Figure 2, this resulted in a significant increase in MOR binding that was detected in the frontal and cingulate cortex, as well as in the caudate putamen, of chronic cocaine-treated rats and those undergoing long-term withdrawal after an escalating dose "binge" cocaine administration paradigm in chronic cocaine-treated rats. This up-regulation in mRNA levels was shown for the first time to persist at least 14 days into withdrawal. Interestingly, the increase in MOR mRNA levels was also observed in the frontal cortex of 3 hours withdrawn rats. However, naloxone in this study had no effect on the increase of MOR mRNA levels. The results indicated that the opioid system was altered during early withdrawal from chronic cocaine administration. Still, the mechanism of MOR up-regulation in escalating-dose chronic “binge” cocaine-treated and cocaine-withdrawn animals remain unknown. The
authors concluded that changes in MOR expression might contribute to the long-term neuroadaptations observed. Further investigations on the MOR is an important manner.

**Figure 2:** Persistent upregulation of MOR in brains of long-term withdrawn escalating dose ‘‘binge’’ cocaine-treated rats. Color autoradiograms of adjacent coronal brain sections showing μ opioid receptor binding in the brains of chronic saline-treated, cocaine-treated, and withdrawn rats. MOR were labeled with a specific dye. The sections shown are from the level of the caudate (Bailey et al., 2005).

Previous studies indicated that steady-state methadone exposure blocked cocaine seeking, as well as cocaine-induced up-regulation of MOR mRNA in the NAc (Leri et al., 2006). The effect of cocaine on MOR mRNA was consistent with several other reports of cocaine-induced alterations of the endogenous opioid system (Winick-Ng et al., 2012; Azaryan et al., 1998; Cohen et al., 1991; Hurd et al., 1992; Izenwasser et al., 1996; Spangler et al., 1993; Turchan et al., 2002; Unterwald 2001). Furthermore, because the changes in MOR mRNA expression were selectively found in neural areas involved in the regulation of incentive motivation and addictive behaviors (Di Chiara 1995; Koob 1992; Wise 1987), those findings provided further evidence for a role of cocaine-induced modification of MOR function in the development and maintenance of cocaine seeking (Gorelick et al., 2005; Kreek 1996; Schroeder et al., 2007; Zubieta et al., 1996). Recently, a review study also indicated that animal models and basic clinical research have shown important interactions between the dopaminergic and the opioid system (Picetti et al., 2013). Also, recent studies showed
that prolonged exposure to high-efficacy agonists for the MOR resulted in desensitization of the receptor. Desensitized receptors are thought to be unable to couple to G-proteins, preventing downstream signaling; however, the changes to the receptor itself are not well characterized (Birdsong et al., 2013), this study will focus on the pathways that are activated downstream to the MOR following exposure to cocaine.

In conclusion, most of the studies showed that the persistent elevation of MOR expression levels observed during cocaine use in rat models, implies an important role of the opioid system, MOR specifically, in cocaine craving and relapse. Also, compounds that modulate the opioid system may be beneficial in treating cocaine addiction (Picetti et al., 2013).

**Cocaine and the mu opioid receptor in humans**

A previous study investigated the relationship between the period of cocaine abstinence and MOR binding to carfentanil (a selective MOR agonist) in regions of the brains of former cocaine addicts. Results showed short interval time to relapse to cocaine was associated with an increased in MOR binding in frontal and temporal cortical regions in the brain at one and 12 weeks of abstinence from cocaine, with a lesser decrease in binding between one and 12 weeks as time passes after abstinence. There were also significant positive correlations between MOR binding at 12 weeks and the percentage of cocaine use days during the first month after relapse. Therefore, MOR binding contributed significantly to the prediction of time to relapse, even after accounting for other clinical variables.
The increased brain MOR binding in frontal and temporal cortical regions is shown in Figure 3 (A & B) (Gorelick et al., 2008). Moreover, recent studies concluded that polymorphisms in MOR are also relevant for cocaine addiction in the African American population and provides additional support for the broad role for MOR in cocaine addiction (Crist et al., 2013).

**Figure 3:** Brain MOR binding and the relationship to relapse to cocaine use after monitored abstinence. (A) Demonstrates the correlation of regional brain mu-opioid receptor (MOR) binding to carfentanil after one week or 12 weeks of enforced abstinence with time to relapse to cocaine use in 15 cocaine-dependent subjects. Brain regions (in red) showing a significant positive correlation between ΔmOR binding (week 1 – week 12) and time to relapse. (B) The relationship between MOR binding (Y axis) after one week or 12 weeks of abstinence and log (time to relapse) (X axis) (Gorelick et al., 2008).

**The effect of cocaine exposure on MOR protein expression in vitro**

Very few studies have examined the direct effects of cocaine *in vitro* and the results have been contradictory. In contrast to *in vivo* findings, in primary cortical astrocyte cultures, exposure to cocaine for 2 or 5 h resulted in decreased MOR mRNA levels (Festa et al., 2002). Previously in the Kalisch laboratory the *in vitro* administration of cocaine was found to increase MOR protein levels in PC12 cells.
(Winick-Ng et al., 2012), which supports the in vivo findings described above. The study by Winick-Ng et al., also provided insight into the mechanisms through which cocaine alters MOR levels, including that the increase may be regulated transcriptionally and post-transcriptionally by nitric oxide (NO) as well.

The effects of cocaine on MOR protein levels were evaluated in extracts of control and cocaine-treated PC12 cells using western blot analysis. Two treatment paradigms were used cells treated with 10, 100 or 500 μM either with a single continuous dose or through repeated intermittent cocaine treatments for 72 h. Densitometric analysis of western blots, revealed a significant increase in MOR protein levels relative to control in PC12 cells exposed to 500 μM cocaine continuously or following exposure to 100 μM cocaine for the repeated intermittent treatment (RIT) paradigm (Winick-Ng et al., 2012).

Winick-Ng and investigators showed that repeated intermittent doses of cocaine also elevated MOR mRNA levels, while a single dose did not, indicating that multiple doses of cocaine are necessary for the transcriptional regulation of the MOR. Both treatment paradigms increased MOR protein stability, indicating that either single or multiple doses of cocaine regulates MOR levels post-transcriptionally (Winick-Ng et al., 2012). Also, the investigation of the role of NO in regulating MOR levels was executed (see below). Since 500 μM single continuous and 100 μM repeated intermittent doses of cocaine increased MOR levels, these treatments were used in the present study.
**The role of Nitric oxide in the cocaine induced MOR expression**

NO has previously been shown to contribute to behavioral effects of cocaine associated with MOR, including conditioned place preference and sensitization (Liddie et al., 2013; Itzhak et al., 1998; Martin and Itzhak 2000; Collins and Kantak 2002; Pudiak and Bozarth 2002; Itzhak 2008). Past studies proved the important role of NO in synaptic plasticity and that reduced brain NOS activity blunts the processes that underlie the development of sensitization to cocaine (Itzhak 2009). In addition, cocaine administration has been reported to increase levels of NO in the rat brain (Sammut and West, 2008; Lee et al., 2010). Winick-Ng at al., 2012, also assessed the NO modulation of MOR in vitro in the PC12 cells, by examining the effects of cocaine on NO production visually in living cells using diaminofluorescein diacetate (DAF-2) fluorescence (Kalisch et al., 2002). Following 72 h of treatment with 100 µM repeated intermittent or 500 µM single continuous cocaine treatment, NO production, as indicated by DAF-2 fluorescence, was clearly enhanced relative to control (untreated) cells. This cocaine-mediated increase in DAF-2 fluorescence was prevented in PC12 cells pre-treated with 20 mM NOS inhibitor Nω-nitro-L-arginine monomethylester (L-NAME). As indicated previously, the results also showed that pretreatment of PC12 cells with L-NAME attenuated the increase in MOR protein levels induced by both single continuous or repeated intermittent cocaine treatments, suggesting NO modulates the post-transcriptional regulation of the MOR receptor.

Interestingly, L-NAME also prevented the increase in MOR mRNA levels associated with repeated intermittent cocaine exposure that suggested NO could also modulate MOR transcription. Increases in neuronal NO production were observed following in vivo administration of cocaine, which resulted from an increase in
neuronal NOS (nNOS) protein levels and activation (Sammut and West 2008; Lee et al., 2010). Although these effects were attributed to alterations in dopamine and glutamate receptor signaling cascades (Sammut and West 2008; Lee et al., 2010), pre- and post-synaptic connections are not present in the PC12 cell culture system suggesting that cocaine may also directly affect NOS levels and/or activity to directly modulate MOR expression.

Moreover, studies showed that repeated exposure to cocaine up-regulates NO efflux into the dorsal striatum and requires nNOS activation, as proposed in Figure 4 (Lee et al., 2010).

Figure 4: A postulated diagram illustrating intracellular mechanisms underlying repeated cocaine-regulated NO efflux in the dorsal striatum. DA dopamine, Glu glutamate, D1R and D2R, dopamine D1 and D2 receptors, mGluRI and mGluRIII groups I and III mGluRs, p phosphorylation. Solid arrows and blunt arrows represent stimulation and blockade of downstream targets, respectively, while broken arrows represent indirect stimulation of p-nNOS via NMDA receptor-coupled Ca+2 signaling cascades (Lee et al., 2010).
Studies have suggested the possibility that dopamine and NO are linked with several signaling pathways including protein kinase C (PKC) (Lee et al., 2010) and the extracellular signal-regulated kinase (ERK) pathway (also known as the mitogen-activated protein kinase pathway (MAPK)) (Chalimoniuk et al., 2009). Therefore, cocaine-mediated up-regulation of MOR expression in the PC 12 cell line may involve activation of multiple signalling pathways.

Summary of possible mechanisms of cocaine's actions within the neuron in literature

Cocaine's mechanism of action within the neuron and it's observed effects remain unclear. Though, there are some proposed mechanism of cocaine in initiating it's effects in the literature. First, the blockade of sodium channels following passive entry of cocaine into PC12 cells is one possibility. However, this may not be the only mechanism because, in PC12 cells, NO activity is associated with extracellular signal-regulated kinase (ERK) pathway activation (Kim et al., 2003; Riccio et al., 2006; Miyamoto et al., 2011), and Tan et al. (1999) found that the selective sodium channel inhibitor tetrodotoxin had no effect on this pathway. The Kalisch laboratory previously determined that NO modulates ERK activity in PC12 cells (Kalisch et al., 2003), and since blockade of ERK phosphorylation in the NAc of rats inhibits cocaine-induced behavioural sensitization (Kim et al., 2011), it is possible that in our system NO-mediated activation of ERK contributes to cocaine-induced MOR expression. As demonstrated previously, in vivo cocaine exposure increases dopamine accumulation, through inhibition of dopamine reuptake by the dopamine transporter, and the dopamine transporter inhibitor sydnocarb has been shown to increase NO generation (Bashkatova et al., 2002). Interestingly, Imam et al. (2005) showed that
cocaine, which increased Nuclear Factor-light chain kappa-enhancer of activated B cells (NFκB) expression, dose-dependently decreases both dopamine transporter expression and intracellular dopamine concentrations in differentiated PC12 cells. Elevations in extracellular dopamine could result in dopamine receptor activation, and inhibition of the D1 sub-type of dopamine receptor was reported to partially inhibit the cocaine-induced increase in NF-κB (Lepsch et al., 2009). Because NF-κB has also been implicated in regulating NOS expression and NO production (Broadbelt et al., 2009; Broadbelt et al., 2011) indirect activation of the D1 receptor by cocaine could enhance NO production. Whether the increases in NO observed in the current study resulted from cocaine-induced dopamine accumulation and dopamine receptor activation requires further investigation. Finally, cocaine has also been reported to diffuse through the membrane of cells (Rang et al., 2001), which suggests that it may exert direct effects on gene or protein expression.

A summary of the different possible pathways involved in cocaine-mediated MOR upregulation

Cocaine maybe linked to the main signaling pathways in the cell; phosphatidylinositol-3 (PI3) kinase - Akt, mitogen activated protein kinase (MAPK) and phospholipase C – protein kinase C (PKC), which regulates various transcription factors and ultimately gene expression. C-Fos is a transcription factor that serves as an indicator of neuronal activity in the central nervous system. Evidence has shown that induction of c-Fos is one of the key steps to form drug-induced neuroadaptations in the cell (Nestler, 2001; J. Zhang et al., 2006). Several signaling pathways have been identified to participate in cocaine-induced c-fos expression, such as protein kinase A (PKA) and PKC (Suh et al., 2004). Also, multiple repeated treatments with cocaine
cause increases in the levels of c-Fos mRNA (Winick-Ng et al., 2012). It was therefore proposed that c-Fos can bind to Jun transcription family members and up-regulates MOR mRNA levels through enhanced transcription (Winick-Ng et al., 2012).

In addition, ERK activation, which is crucial for cell responses to changes in the environment (Schaeffer et al., 1999), was reported to be activated after acute cocaine treatment in the brain (Valjent et al., 2006).

Figure 5 is a demonstration of the different signaling pathways that cocaine may modulate, and the relationship between NO and these pathways. The present study will investigate the role of these signaling pathways and their relationship to NO in cocaine-induced MOR expression in the PC12 cell line.
Figure 5: Schematic representation of the proposed mechanisms for the in vitro action of cocaine on MOR expression via different signaling pathways, and Nitric Oxide (NO). Cocaine activates 3 main signaling pathways - the phosphoinositol – 3 (PI3) kinase – Akt, Ras-MAPK and Protein kinase C (PKC). These pathways in turn affect downstream signaling and expression of transcription factors leading to altered gene expression. Nitric Oxide (NO) affects the activation of these pathways however; the mechanism and specific effects are yet to be fully elucidated.

The PKC pathway

Protein kinase C (PKC) is a family of enzymes that modify protein function through phosphorylating serine and threonine residues on modified proteins. This family consists of at least ten isozymes (Mellor and Parker, 1998). In mammalian cells, including neurons, PKC is one of most ubiquitous multifunctional kinases involved in the modification of a variety of substrate proteins related to diverse cellular and synaptic activities (Dempsey et al., 2000; Geraldes and King,
2010; Rosse et al., 2010). Accumulative data have demonstrated an essential role of PKC in drug action (Newton and Ron, 2007; Lee and Messing, 2008; Olive and Newton, 2010), although the direct effect of cocaine on the PKC pathway is yet to be clarified. Previous study concluded that cocaine disrupts signal transduction in PC 12 cells by altering the expression and activity of PKC isoforms in vitro (Onaivi et al., 1998). Recent studies also examined the possible impact of cocaine on PKC maturation in the rat striatum in vivo (Xue et al., 2012). This study concluded that cocaine increased PKC phosphorylation in the rat NAc and caudate putamen in a dose-dependent manner in vivo. Cocaine also up-regulated PKC phosphorylation in the medial prefrontal cortex and PKC phosphorylation was transient in a time-course fashion, suggesting that cocaine dosage regimens affect the PKC activation profile.

Moreover, both NO donors and peroxynitrite can activate PKC isoforms (Bapat et al., 2001; Liang and Knox 1999) suggesting that NO could be altering PKC signal transduction in PC12 cells to regulate MOR expression. Also, it seems to be that the modulation of the dopaminergic receptors by cocaine exposure affected NO efflux in vivo which involved the PKC pathway, as mentioned previously (Lee et al., 2010). This study will explore this probable pathway in the up-regulation of the cocaine-mediated MOR levels in the PC 12 cell line.

The MAPK/ERK pathway

Radwanska and his colleagues showed that ERK was involved in c-Fos expression induced by chronic (but not acute) cocaine exposure in the mouse amygdala (Radwanska et al., 2005; Guan et al., 2008). In contrast, results showed that inhibition of ERK activation by SL 327 (its inhibitor) could significantly attenuate c-
Fos protein expression induced by acute cocaine exposure in the NAc. This suggested that ERK might be a critical mediator for acute cocaine-induced c-Fos protein expression in the NAc (Guan et al., 2008). The acute cocaine-induced c-Fos expression has been observed in both the core and the shell of the NAc, but inhibition of ERK activation affected cocaine-induced c-Fos protein mainly in the core region of NAc (Ito et al., 2000).

The authors concluded that both core and shell in the NAc play a significant role in cocaine addiction and relapse, but ERK signaling is only involved in the cocaine-induced expression of c-Fos in the core. Whether similar differences occur with respect to MOR expression is not known.

Furthermore, it was determined previously that NO modulates ERK activity in PC12 cells (Kalisch et al., 2003), and since blockade of ERK phosphorylation in the NAc of rats inhibits cocaine-induced behavioral sensitization (Kim et al., 2011), it is possible that in PC12 cells, NO-mediated activation of ERK contributes to cocaine-induced MOR expression.

The Akt pathway

The PI3 kinase pathway leads to the phosphorylation of the serine/threonine kinase Akt, also known as protein kinase B (PKB), which has been implicated in many cellular processes. Akt has been shown to be involved in regulating cell proliferation, survival and growth, as well as enhancing the expression of transcription factors and endothelial nitric oxide (eNOS) (Brazil, Park, and Hemmings, 2002; Rodgers and Theibert, 2002). Previous studies suggested that since
the administration of amphetamine resulted in an inhibition of Akt phosphorylation/activation signaling pathway, and the Akt pathway is regulated through dopaminergic receptors, the Akt pathway participates in psychostimulant-induced behavioral activation such as cocaine (Beaulieu et al., 2005; Chen et al., 2009; Self et al., 1995). Also, recent studies indicated cocaine effects on Akt phosphorylation were inhibited by pre-incubation with the phosphatidylinositol 3-kinase inhibitor LY294002, suggesting that the Akt pathway is a critical mediator of cocaine-induced structural plasticity in vivo, suggesting the involvement of this pathway in cocaine addiction (Collo et al., 2012).

Moreover, NO has also been found to modulate the activation of Akt in PC12 cells (Li et al., 2006; Cragg et al., 2012), suggesting that this pathway could also be involved in the regulation of MOR expression by cocaine.

The exact mechanisms through which cocaine and NO modulate MOR levels still remain unclear. Therefore, in this study, the role of PKC, ERK and Akt activation in the cocaine-induced up-regulation of the MOR in the PC 12 cell line was examined. In addition, whether or not NO is linked to a specific pathway was also assessed. The findings in this study will broaden our knowledge in understanding the mechanisms involved in cocaine-mediated MOR up-regulation.
RATIONALE

Relapse is one of the main challenges facing the current treatment of cocaine addiction. Understanding its neurobiological mechanisms through which cocaine produces changes in the central nervous system is a critical step toward developing effective anti-relapse therapies. Since cocaine users have increased regional brain MOR binding which correlates with cocaine craving, the role of the opioid system and the MOR is currently being investigated.

The endogenous opioid systems and cocaine addiction are linked on many levels (Shippenberg et al., 2007; Roth-Deri et al., 2008; Cummins and Leri 2009; Kreek et al., 2009; Wee and Koob 2010). Of interest are the genomic effects of cocaine administration, including increased peptide expression levels of MOR (Cohen et al., 1991; Azaryan et al., 1996; Unterwald 2001; Zhou et al., 2002, 2010; Bailey et al., 2005), which occur in regions of the brain known to regulate incentive motivation and stress reactivity (DiChiara 1995; Wise 1996; Koob & Kreek 2007).

In rat models, cocaine-induced increases in MOR mRNA expression have been consistently observed in the ventral striatum (Leri et al., 2006, 2009). Furthermore, positron emission tomography (PET) studies in abstinent cocaine users have established correlations between elevations in MOR binding in mesocorticolimbic areas and intensity of cravings (Zubieta et al., 1996; Gorelick et al., 2005; Ghitza et al., 2010). Because links between cocaine exposure and alterations in MOR expression/function are established, the aim of the present study was to elucidate the molecular mechanisms involved in the regulation of the MOR by cocaine.
In PC12 cells, multiple intermittent cocaine treatments significantly increased NO production as well as MOR mRNA and protein levels and the NOS inhibitor, L-NAME prevented the cocaine-induced increases in MOR protein and mRNA levels (Winick-Ng et al., 2012). Cocaine has been reported to activate a number of signaling pathways including ERK, Akt and PKC (Collo et al., 2012; Radwanska et al., 2005; Guan et al., 2008; Onaivi et al., 1998), however the role of these pathways in the cocaine-induced increase in MOR levels in vitro is not known.

Therefore, the present study will assess the effects of inhibitors of the individual signaling pathways on the cocaine-induced increase in MOR protein levels in PC12 cells. The inhibitors used will include the PLC-PKC inhibitors U-73122 and BIS-1, and the MAPK pathway inhibitor U0126, and the LY94002 inhibitor was used to investigate the PI3K/Akt pathway as these treatments have been found to modulate protein expression in PC12 cells (Binnington and Kalisch, 2007). These three signaling pathways have also been reported to be modulated by NO (Cragg et al., 2012; Kalisch et al., 2003; Bapat et al., 2001; Liang and Knox 1999), however the exact link between these signaling pathways and NO in cocaine-treated PC12 cells has not been investigated previously. Thus, the effects of signaling pathway inhibitors on cocaine-induced NO production and the effects of NOS inhibition on signaling pathway activation in PC12 cells was also investigated.

Hypothesis

Cocaine and NO modulates the MOR through multiple signaling pathways.
Objectives

The present study used PC12 cells, which were particularly valuable because: 1) the MOR gene is expressed in PC12 cells (Borowitz et al., 1997; Yoshikawa et al., 1999; Niu et al., 2000; Winick-Ng et al., 2012); 2) these cells have been used previously to examine the role of NO in gene expression (Kalisch et al., 2002, 2003; Binnington and Kalisch, 2007; MacKinnon et al., 2012; Cragg et al., 2012); and 3) the role of PKC, Akt and ERK signaling pathways in gene expression in this cell line have been well characterized (Mackinnon et al., 2012; Binnington and Kalisch, 2007; Kalisch et al., 2003; Cragg et al., 2012).

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), horse serum, gentamycin, penicillin, streptomycin, DNase I, Oligo dT, Superscript II, primers, and Platinum Taq were purchased from Invitrogen (Mississauga, ON, Canada) and fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT, USA). Cocaine HCl was from Dumex (Toronto, ON, Canada), Nω-nitro-L-arginine methyl ester (L-NAME), and mouse monoclonal anti-α-tubulin were purchased from Sigma Aldrich (St. Louis, MO, USA). 4,5-Diaminofluorescein diacetate (DAF-2 DA) and the inhibitors bisindoylmaleimide 1 (Bis-1), U 73122, LY 294002, and actinomycin D inhibitor were obtained from Calbiochem® EMD Chemical, Inc. (Gibbstown, USA) and U 0126 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The complete mini tablets were purchased from Roche Diagnostics (Laval, QC, Canada), the sodium dodecyl sulfate (SDS) sample buffer, DTT, and protein
standards were obtained from New England Biolabs (Ipswich, MA) and the polyclonal MOR antibody was from Abcam (Cambridge, MA, USA) or Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Luminol was also purchased from Santa Cruz. Hybond-C blotting membranes, sheep anti-mouse IgG and enhanced chemiluminescence (ECL) kit were obtained from Amersham/GE Health Care (Piscataway, NJ, USA), poly-D-lysine was from BD Biosciences (Mississauga, ON, Canada). Syber Green PCR master mix was obtained from Qiagen (Toronto, ON, Canada). All other chemicals were molecular or electrophoresis grade and obtained from Fisher Scientific (Ottawa, ON, Canada) or DiaMed Laboratories (Mississauga, ON, Canada). Rat pheochromocytoma cells (PC12 cells) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA).

**Cell culture and treatments**

Rat PC12 cells were maintained in DMEM containing 5% FBS, 5% horse serum and 50 mg/mL gentamycin at 37°C in 5% CO₂. To evaluate the effects of cocaine alone or in combination with various inhibitors on MOR protein levels, cells were plated on Corning® 35 mm 6 well plates at a density of 5.0 x 10^5 cells per plate or Corning® 60mm dishes at a density of 1.0 x 10^6 cells per plate for protein.

To evaluate the effects of cocaine and various inhibitors on NO production PC12 cells were plated on 6-well culture dishes containing poly-D-lysine coated cover-slips at a concentration of 2.0 x 10^5 cells per well.

The effects of cocaine were determined by exposing the cell lines to various concentrations of cocaine using two different treatment regimens. The doses of
cocaine selected for this study (100, and 500 µM) were based on previous reports investigating the effects of cocaine on morphological changes and proto-oncogene expression (Imam et al., 2005) and increased MOR expression (Winick-Ng et al., 2012) in PC12 cells. Two treatment regimens were chosen based on previous findings indicating that different exposure patterns can differentially affect MOR binding affinity and receptor density in several regions of the rat brain (Unterwald et al., 1993; Izenwasser et al., 1996) and MOR protein levels in PC12 cells (Winick-Ng et al., 2012). In the present study, cells were either exposed to a single continuous treatment \((SCT)\) or repeated intermittent treatments \((RIT)\) with cocaine (see Table I). The dose of cocaine for the \(SCT\) was 500 µM, and involved treatment of the cells with one dose of cocaine on day 0 and the cells were then left in the incubator until they were to be harvested after 72 h (day 3). The \(RIT\) dosing regimen included 3 daily treatments of 100 µM cocaine, 30 min each, separated by 60 min exposures to cocaine-free media. Cells were harvested 72 h after treatment began, except where otherwise indicated. For inhibitor studies (see below), the media used to feed cells between cocaine treatments contained inhibitors.
<table>
<thead>
<tr>
<th>Regimen</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SCT</strong></td>
<td>A single dose of cocaine added to the cell culture media and not removed over the entire time-course (72 h)</td>
</tr>
<tr>
<td><strong>RIT</strong></td>
<td>Three daily intermittent 30 min treatments applied to the cells separated by 1 h of regular cell culture media.</td>
</tr>
</tbody>
</table>

**Table I**: Cocaine treatment regimens. PC12 cells were either given a single continuous treatment (SCT) or a repeated intermittent treatment (RIT) of cocaine.

In order to determine the specific pathway that cocaine acts through, the cocaine mediated MOR up-regulation was examined by treating PC12 cells with inhibitors for the several pathways. The pathways examined were: PLC-protein kinase C (PKC), PI3-Akt and mitogen activated protein kinase (MAPK) signaling pathways. Cells were plated onto 35 mm 6 well dishes 24h prior to any treatment. Media was always changed immediately before beginning a treatment time course for SCT and RIT, and the inhibitors were added directly to the culture media. For the initial treatment inhibitors were added 2 h prior to the addition of cocaine. For RIT, cells were fed with fresh media and retreated with inhibitors and cocaine after 24, 48 and 72 h with inhibitors being added 1 h before treatment with cocaine. Treated cells were rinsed with ice-cold PBS and harvested in specified buffers after aspirating media at the end of the treatment course for protein (72 h). Untreated or inhibitor only treated PC12 cells were used as controls.

The doses of the inhibitors used were based on previous findings in PC12 cells and are summarized in table II. To examine the role of the PI-3 kinase-Akt pathway,
cells were pre-treated with 10 µM LY294002. This dose was chosen as it was previously found to inhibit activation of Akt in PC 12 cells resulting from 1 h of 50 ng/mL NGF administration (unpublished results). This dose of LY294002 also prevented the NGF-mediated increase in amyloid precursor protein (APP) levels (Binnington and Kalisch 2007).

The NGF-induced activation of MAPK was inhibited by pretreatment of PC12 cells with 50 µM of the MEK1/2 inhibitor U0126 (Kalisch et al., 2003). This dose also prevented the NGF-induced increase in APP protein levels (Binnington and Kalisch, 2007) and partially reduced the NGF-mediated increase in lipoprotein receptor-related protein promoter activation (Grana et al., 2013).

The role of phospholipase C-PKC pathway was also evaluated using inhibitors U73122 and bisindolylmaleimide 1 (BIS-1). BIS-1 shows high selectivity for PKC-α, βI, βII, γ, 9 and Ɛ isozymes, while U 73122 is an inhibitor of phospholipase-C an upstream activator of PKC and other molecules.

NOS was inhibited by pre-treating PC 12 cells with 20 mM L-NAME. This dose prevented the NGF-mediated increases in NOS activity, NO production and ChAT mRNA expression and specific activity (Kalisch et al., 2002) and was shown to be effective in preventing the cocaine-induced up-regulation of MOR expression in the PC 12 cell line (Winick-Ng et al., 2012).
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Pathway</th>
<th>[Stock]</th>
<th>Vehicle</th>
<th>[Final]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY294002</td>
<td>PI3-Akt</td>
<td>10 mM</td>
<td>DMSO</td>
<td>10 µM</td>
</tr>
<tr>
<td>U0126</td>
<td>MEK-MAPK</td>
<td>10 mM</td>
<td>DMSO</td>
<td>50 µM</td>
</tr>
<tr>
<td>BIS-1</td>
<td>PKC-α, βI, βII, Y, 9 and ε isozyymes</td>
<td>2.5 mM</td>
<td>DMSO</td>
<td>10 µM</td>
</tr>
<tr>
<td>U-73122</td>
<td>PLC-PKC</td>
<td>0.5 mM</td>
<td>Ethanol</td>
<td>5 µM</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NOS</td>
<td>2 M</td>
<td>Water</td>
<td>20 mM</td>
</tr>
<tr>
<td>Act D</td>
<td>transcription</td>
<td>-</td>
<td>Ethanol</td>
<td>1 µg/ mL</td>
</tr>
</tbody>
</table>

**Table II:** Inhibitors used to evaluate the role of various signaling pathways, transcription and NO in the cocaine-induced regulation of MOR expression.

**Figure 6:** Representative diagram of the study design. The different pathways: PLC-PKC pathway, MAPK pathway, and the PI3-Akt pathway are tested by adding specific pathway inhibitors: U73122 and BIS-1 for inhibition of the PLC-PKC pathway, U0126 for inhibiting the MAPK pathway, and LY294002 for inhibiting the PI3K-Akt pathway by using western blot analysis.
Western immunoblot analysis

Control and treated PC12 cells were lysed in 150 µL of radioimmunoprecipitation assay (RIPA) buffer per 35 mm well of the 6-well plates or 250 µL RIPA buffer for 60 mm plates (final concentration: 50 mM Tris, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.5% SDS, 1 mM each of EDTA, sodium fluoride, sodium orthovanadate, and protease inhibitor [one Complete Mini tablet (Roche Diagnostics)/10 mL], pH 7.4). Samples were rocked on ice for 15 min, sonicated and centrifuged at 17 530 g for 15 min. The protein content of the supernatant was determined by the method of Bradford (1976). Cell lysates (100 µg) were then boiled in SDS sample buffer (final concentration: 62.5 mM Tris-HCl; pH 6.8, 2% SDS, 42 µM DTT, 10% glycerol and 0.01% phenol red) and loaded onto a 10% SDS/polyacrylamide gel. Each lane contained the same final volume based on calculations to ensure the right quantity of protein (100 µg) followed by adjustment of the volume with the addition of deionized water and the appropriate amount of dye.

Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Hybond-C) using a Trans-blot semidry transfer unit (Bio-Rad Laboratories, Mississauga, ON, Canada) with transfer buffer (final concentration: 28 mM Tris, 39 mM glycine and 20% methanol, pH 9.2). Membranes were blocked in 2% of non-fat milk in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 h in order to eliminate unwanted background bands. Blots were then incubated in 1:750 rabbit MOR antibody in 1% bovine serum albumin (BSA) in TBS-T for 2 h (Abcam), or in 1:200 rabbit MOR antibody in 1% non-fat milk in TBS-T (Santa Cruz) overnight. Antibody detection was achieved using 1:2500 horseradish peroxidase-
conjugated donkey anti-rabbit IgG in either 1% BSA or 5% non-fat milk in TBS-T for 1 h, followed by exposure to ECL for 5 min or luminol for 1 min.

Membranes were scanned using the STORM 860 (Molecular Dynamics, subsidiary of Amersham) for ECL, or the Fluorchem 9900 imaging system (Alpha Innotech, Santa Clara, CA, USA) for luminol. Bands were analyzed densitometrically using Imagequant (Molecular Dynamics) or Fluorchem 9900 software. After detecting MOR bands, blots were stripped with 62.5 mM Tris, pH 6.7, containing 2% SDS and 100 mM 2-mercaptoethanol at 50°C for 20 min. Membranes were then rinsed in TBS for at least 4 h before blocking with 5% milk in TBS-T for 1 h and reprobing with 1:50 000 mouse monoclonal anti-α tubulin antibody overnight. Blots were then exposed to 1:2500 goat anti-mouse IgG-horse radish peroxidase conjugated secondary antibody, in 5% milk in TBS-T and the protein bands visualized as described above.

**Analysis of NO production**

NO production was assessed using the fluorescent probe DAF-2. PC12 cells were treated with cocaine (100 RIT or 500 μM SCT) in the presence or absence of the inhibitors for 3 days, washed once with media and loaded with 10 μM DAF-2 in 1 mL culture media. Following 2 h of incubation cells were washed 4 times with 2 mL media, and DAF-2 fluorescence was visualized using an Olympus IX-81 fluorescence microscope (excitation at 488 nm, emission at 520 nm) with an Olympus LucPlan FL 0.40 aperture lens (at 20 x magnification) in phosphate buffered saline at room temperature. Digital images were captured using a Cascade 512F camera (Photometrics, Tucson, AZ, USA), and processed in Q-Capture and Adobe Photoshop 5.0.
Data Analysis & Statistics

For western blot analysis, each band was analyzed densitometrically and expressed as a percentage of the total amount of that protein present on the entire blot. MOR protein levels were then normalized to α-tubulin levels within the same sample.

Data are representative of at least 5 independent experiments, unless otherwise indicated, and are presented as the mean ± standard error of the mean (SEM). Data were assessed for normality and homogeneity of variance and statistical analysis was carried out using a one-way analysis of variance (ANOVA). In a one-way ANOVA, analysis was followed by Dunnett’s test or the Tukey-Kramer multiple comparisons test to determine which groups were significantly different, or by students t-test when comparing two treatment groups. This data analysis was carried out using GraphPad InStat software for analyzing the raw data. Mean values were considered different if p<0.05.
RESULTS

The effects of cocaine on MOR expression

The effects of cocaine on MOR protein levels were evaluated in extracts of control and treated PC12 cells using western blot analysis. A representative immunoblot depicting MOR protein levels (upper panel) and the corresponding α-tubulin levels (lower panel) obtained from cells treated with a SCT 500 μM, and RIT 100 μM cocaine for 72 h is shown in Figure 7A respectively. Densitometric analysis revealed a significant increase in MOR protein levels in SCT (Figure 7B) and RIT (Figure 7C) cocaine relative to SCT and RIT control (*P<0.001; n=5, for both). These two dose/treatment paradigms were used in subsequent experiments.

A

<table>
<thead>
<tr>
<th>Cocaine (μM)</th>
<th>SCT control</th>
<th>SCT 500</th>
<th>RIT control</th>
<th>RIT 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>45 kDa</td>
<td>55 kDa</td>
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Figure 7: Effect of cocaine on MOR expression levels via SCT or RIT. (A) Representative western blot of MOR protein expression (upper blot) and tubulin (lower blot) following treatment of PC12 cells with 500 µM SCT and 100 µM RIT. The lanes were loaded as follows starting: SCT control, SCT cocaine, RIT control, RIT cocaine. (B) Densitometric western blot analysis of MOR expression levels in response to SCT cocaine treatment (500 µM) in PC 12 cells normalized to tubulin levels. There was a statistically significant increase in MOR expression level after the cells were treated with a SCT of cocaine in comparison to the first column of untreated cells (SCT control) *P<0.001. (C) Densitometric western blot MOR expression levels analysis in response to RIT cocaine treatment (100 µM) in PC 12 cell line. RIT cocaine up-regulated MOR expression levels in comparison to RIT control untreated cells; *p< 0.001. Statistical analysis was conducted using one way
ANOVA followed by student t-test for significance between the two columns. Results are shown as mean ± SEM. Analysis was performed after at least five independent replicates of the same experiment. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

The effect of cocaine-induced increase in MOR expression level on transcription

To determine whether cocaine increased MOR expression by altering transcription, cells were pretreated with actinomycin D, which inhibits transcription from occurring by preventing RNA polymerase from interacting with DNA by binding to dG residues (Sobell, 1985). PC 12 cells were treated with 1 µg/mL actinomycin D (Binnington and Kalisch, 2007) to study the effects of cocaine-mediated transcription on MOR protein expression levels. The levels of MOR protein expression were determined by western blot analysis (Figure 8A).

As apparent from Figure 8B, densitometric analysis revealed that actinomycin D significantly reduced the levels of MOR protein expression after 100 µM RIT of cocaine (**P<0.001).

A

MOR

α-tubulin

45 kDa

55 kDa

RIT control  RIT 100  RIT 100 + +

Cocaine (µM)

Actinomycin D (1 µg/mL)
Figure 8: Effect of RIT cocaine-induced MOR expression levels on transcription. (A) Representative western blot of MOR protein expression (upper blot) and tubulin (lower blot) following treatment of PC12 cells with 100 µM RIT, in the presence or absence of 1 µg/mL actinomycin D. The lanes were loaded as follows starting with lane 1: RIT control, RIT cocaine, RIT actinomycin D inhibitor, RIT actinomycin D inhibitor+cocaine. (B) Densitometric analysis of MOR protein levels relative to tubulin; the effect of RIT cocaine administration on transcription by the addition of actinomycin D inhibitor (1 µg/mL). RIT control PC 12 cells without the addition of cocaine nor inhibitor and RIT cocaine administration, at dose of 100 µM, resulted in a statistically significant increase in relative MOR levels(*P<0.001). MOR levels were not different from control in cells treated with actinomycin D in either the presence or absence of cocaine and relative to cocaine alone, MOR expression was significantly reduced in cells treated with RIT cocaine and actinomycin D; **P<0.001 for both. Statistical analysis was conducted using one way ANOVA followed by Tukey test for significance between all pairs of columns. Results are shown as mean ± SEM. Analysis was performed after at least five independent replicates. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

Role of the PLC-PKC signaling pathway in the cocaine-induced increase in MOR protein expression levels

The PLC-PKC pathway inhibitors were 5 µM U73122 and 10 µM BIS-1, the effects of these two inhibitors on MOR protein expression levels in cocaine-treated PC 12 cells for both treatment regimens are shown in Figures 9, 10 (A & B) and 11 (A & B). Representative western blot depicting MOR and α-tubulin levels in extracts
obtained from cells treated with cocaine in the presence or absence of PLC-PKC pathway inhibitors, U73122 and BIS-1, for both treatment regimens is presented in Figure 8, and densitometric analysis of cocaine-induced MOR expression relative to α-tubulin for both treatment regimens are depicted in Figures 10 (A & B) for SCT, 11 (A & B) for RIT.

Densitometric analysis revealed there were significant differences between treatments, and treatment regimens. SCT cocaine increased MOR protein levels relative to control levels (*P<0.001, *P<0.01) for BIS-1 and U73122, respectively. Pre-treatment with both BIS-1 and U73122 prior to cocaine SCT significantly decreased MOR levels relative to cocaine treatment alone (***P<0.001, **P<0.01) for both BIS-1 and U73122, as shown in Figures 10A and 10B. There was no difference in MOR levels in samples obtained from control cells and those treated with BIS-1 or U73122 in the presence or absence of cocaine.

MOR protein levels following the RIT treatment regimen with inhibitors of the PLC-PKC signaling pathway are shown in Figures 11 (A & B). Treatment with either cocaine alone or BIS-1 alone significantly increased MOR protein levels relative to control levels (*P<0.05, *P<0.01) respectively, as shown in Figure 11A. Similarly, MOR protein levels following RIT with cocaine or RIT U73122 alone were increased relative to RIT control (*P<0.01, *P<0.05) respectively, as shown in figure 11B. Furthermore, statistical significance occurred between RIT U73122 inhibitor alone with both RIT control and RIT U73122 prior to treatment of cocaine, ( ***P<0.01 for both). Other differences between cocaine-induced MOR levels were considered non-significant.
Figure 9: The effect of cocaine on MOR expression levels in the presence or absence of PLC-PKC pathway inhibitors for SCT and RIT. Western blot of MOR expression (upper blot) and tubulin (lower blot). The lanes were loaded as follows starting with lane 1: SCT control, SCT cocaine, SCT Bis-1, SCT U73122, SCT Bis-1+cocaine, SCT U73122+cocaine, RIT control, RIT cocaine, RIT Bis-1, RIT U73122, RIT Bis-1+cocaine, RIT U73122+cocaine. Doses for cocaine were 500 µM and 100 µM for both SCT and RIT respectively. Tubulin was used as a loading control.
Figure 10: The effect of SCT cocaine on MOR expression levels on the PLC-PKC pathway. (A) Densitometric western blot analysis of MOR expression levels normalized to tubulin levels. There was a statistically significant increase in MOR expression after the cells were treated with a SCT of cocaine (500µM) in comparison to untreated cells (SCT control; *P<0.001). There was no statistically significant difference in relative MOR protein levels between SCT control and SCT(10 µM) BIS-1 alone. MOR protein levels were significantly decreased following SCT with cocaine and BIS-1 compared to cocaine treatment alone (**P<0.001 for both). (B) Densitometric western blot analysis of MOR expression levels, after being treated with a SCT of cocaine (500µM), then normalized to tubulin levels. There was a statistically significant increase in MOR expression level after the cells were treated with a SCT of cocaine in comparison to untreated cells (SCT control) *P<0.01. There was also a statistical significance between SCT cocaine level and with both the SCT inhibitor alone (U73122; 5 µM) and SCT inhibitor + cocaine levels (**P<0.01 for
both). All other pairs of column levels were considered not significant. Statistical analysis was conducted using a one way ANOVA followed by Tukey test for significance between all pairs of columns. Results are shown as mean ± SEM. Analysis was performed after at least five independent replicates of the same experiment. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

**Figure 11:** The effect of *RIT* cocaine on MOR expression levels on the PLC-PKC pathway. (A) Densitometric western blot MOR expression levels analysis; the effect of *RIT* cocaine administration on the PLC-PKC pathway by the addition of BIS-1 inhibitor (10 µM) and after being normalized to tubulin levels. The first column is
the RIT control PC 12 cells without the addition of cocaine nor inhibitor. The second column represents the RIT cocaine administration, a dose of 100 µM which is conceded statistically significant in comparisions to RIT control * P<0.05. There was also a significant increase in MOR expression level between RIT control level and RIT BIS-1(*P<0.01). Other differences between pairs of columns were considered statistically non-significant.(B) Densitometric westren blot MOR expression levels analysis in PC12 cells; response to RIT cocaine in the presents or absence of the inhibitor of the PLC-PKC pathway (U73122; 5 µM). There was a statistically significant difference between RIT control levels and RIT cocaine levels, and RIT U73122 (*P<0.01, *P<0.05) respectively. Also, a statistically significant difference occurred between RIT U73122 with both RIT control and RIT U73122+cocaine with a P value of ***P<0.01 for both. Other differences between pairs of columns were considered statistically nonsignificant. Statistical analysis was conducted using a one way ANOVA followed by Tukey test for significance between all pairs of columns. Results are shown as mean ± SEM. Data is representative of at least five independent assay replicates. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

Effect of U0126 and LY294002 on cocaine-induced increase in MOR protein expression levels

The role of the MAPK and PI3K/Akt pathways was evaluated at first by the addition of pathways inhibitors; U0126 and LY94002, respectively, to PC12 cells in the presence and absence of cocaine treatment. Both SCT and RIT treatment regimens were explored. The effects of these two inhibitors on MOR protein expression levels in cocaine-treated PC 12 cells for both treatment regimens are shown in Figures 12, 13 (A & B) and 14 (A & B). Representative western blot depicting MOR and α-tubulin levels in extracts obtained from cells treated with cocaine in the presence or absence of MAPK and Akt pathway inhibitors, U0126 and LY94002, for both treatment regimens is presented in Figure 12.

Figures 13A and 13B shows a significant increase in MOR protein expression levels following SCT cocaine and RIT cocaine alone relative to SCT and RIT control levels; *P<0.05, *P< 0.01, respectively. There was no significant difference in MOR
protein levels between control cells and those treated with U0126 for the SCT regimen, however, *RIT* U0126 MOR levels were different from *RIT* control levels; *P*<0.05. Pretreatment with U0126 prevented the cocaine-induced increase in MOR protein expression in both treatment regimens with statistical significance of **P**<0.001, and **P**<0.01 for SCT and RIT respectively. Also, SCT U0126 had reduced levels of MOR expression in comparison to SCT cocaine, with a significance of **P**<0.01.

As for the Akt pathway, densitometric western blot analysis revealed that MOR expression levels significantly increased after cells were treated with a SCT cocaine in comparison to SCT control (*P*<0.01). MOR levels were reduced significantly after the pretreatment of PC12 cells with SCT LY 94002 with a significance of **P**<0.05, as seen in Figure 14A. Also, in SCT LY94002 treated cells MOR levels were reduced compared to MOR levels in cells treated with SCT cocaine alone (**P**<0.01).

Moreover, western blot analysis of MOR expression levels for RIT cocaine and RIT LY94002 inhibitor alone were increased in comparison to RIT control (*P*<0.01, *P*<0.05) respectively. Pretreatment of PC12 cells with RIT inhibitor significantly decreased MOR expression levels (**P**<0.001), as shown in Figure 14B.

In conclusion, both pathway inhibitors prevented the up-regulation of MOR protein expression for both treatment regimens. Also, RIT with each inhibitor alone also increased MOR protein levels relative to control levels (*P*<0.05; for both
inhibitors). These results suggest that the MAPK and Akt pathways regulate MOR expression in cocaine-treated PC12 cells.

**Figure 12:** The effect of cocaine on MOR expression levels in the presence or absence of both inhibitors of the MAPK pathway and the PI3-Akt pathway for *SCT* and *RIT* regimens. Western blot of MOR expression (upper blot) and tubulin (lower blot). The lanes were loaded as follows starting with lane 1: *SCT* control, *SCT* cocaine, *SCT* U0126, *SCT* LY94002, *SCT* U0126+cocaine, *SCT* LY94002+cocaine, *RIT* control, *RIT* cocaine, *RIT* U0126, *RIT* LY94002, *RIT* U0126+cocaine, *RIT* LY94002+cocaine. Doses for cocaine were 500µM and 100 µM for both *SCT* and *RIT* respectively. Tubulin was used as a loading control.
Figure 13: Effect of cocaine *SCT* and *RIT* via MAPK/ERK pathway inhibition on MOR protein expression in PC12 cells. (A) Densitometric analysis of MOR protein levels relative to tubulin following 500 µM *SCT* cocaine administration in the presence or absences of the MAPK pathway inhibitor U0126 (50 µM). *SCT* cocaine administration significantly increased MOR protein levels in comparison to the *SCT* control (*P<0.05). There was no difference in in MOR expression levels between control *SCT* inhibitor alone, and *SCT* inhibitor + cocaine. U0126 prevented the cocaine-mediated increase in MOR expression (***P<0.001). *SCT* cocaine and *SCT* U0126 also had a statistical difference of (***P<0.01). (B) Densitometric western blot MOR expression levels analysis in response to *RIT* cocaine treatment (100 µM) in the presents or absence of inhibitor U0126 (50 µM). *RIT* cocaine up-regulated MOR expression levels in comparison to *RIT* control untreated cells; *P< 0.01. MOR expression levels in *RIT* U0126+cocaine were significantly decreased in comparison to *RIT* cocaine alone ***P<0.01. *RIT* control level was statistically different from *RIT*
inhibitor alone (U0126); *P < 0.05. Other differences between pairs of columns were considered statistically nonsignificant. Statistical analysis was conducted using one way ANOVA followed by Tukey test for significance between all pairs of columns. Results are shown as mean ± SEM. Data is representative of at least five independent assay replicates. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

Figure 14: Effect of cocaine SCT and RIT via PI3-Akt pathway inhibition on MOR protein expression in PC12 cells. (A) Densitometric western blot analysis of MOR expression levels, after being treated with a SCT of cocaine (500µM), then normalized
to tubulin levels. There was a statistically significant increase in MOR expression level after cells were treated with a SCT cocaine in comparison to untreated cells (SCT control) *P<0.01. There was also a statistical significant difference in MOR expression levels between SCT cocaine levels with the SCT inhibitor alone (LY294002; 10 µM), and the SCT inhibitor + cocaine, P values were **P<0.01 and *P<0.05 respectively. All other pairs of column levels were considered non-significant. (B) Densitometric westen blot MOR expression levels analysis in PC 12 cells; response to RIT cocaine in the presents or absence of the inhibitor of the PI3K-Akt pathway (LY 94002;10 µM). RIT cocaine (100 µM) significantly increased MOR expression levels in comparison to untreated RIT control samples of PC 12 cells; *P<0.01. Also, there was a significant difference between RIT control and RIT LY94002; *P<0.05. MOR expression levels in RIT LY 294002+cocaine were significantly decreased in comparison to RIT cocaine alone; **P<0.001. Other differences between pairs of columns were considered statistically nonsignificant. Statistical analysis was conducted using one way ANOVA followed by Tukey test for significance between all pairs of columns. Results are shown as mean ± SEM. Data is representative of at least five independent assay replicates. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

**Effect of cocaine on nitric oxide production, and its role in MOR protein expression**

For this study, only the RIT dosing regimen was assessed. Figures 15 and 16 show the role of the various signaling pathway inhibitors in the cocaine-mediated enhancement of NO production in PC12 cells as measured by DAF-2 fluorescence. The effect of cocaine on DAF- 2 fluorescence was evaluated in PC12 cells 72 h after treatment with 100 µM RIT. The MAPK pathway was inhibited using 50 µM RIT U0126, 10µM RIT LY94002 was administered for the inhibition of the Akt pathway, and cells were treated with 5 µM RIT U73122 which is an inhibitor of PLC-PKC pathway activation. As seen in Figures 15 and 16, a low level of DAF-2 fluorescence, indicative of NO production, was observed in untreated PC12 cells (RIT control) and DAF-2 fluorescence appeared to be increased in cells treated with 100 µM RIT cocaine. Treatment with U0126 alone had no effect on DAF-2 fluorescence and when cells were pretreated with U0126, cocaine was still able to increase DAF-2
fluorescence (Figure 15). In contrast, pretreatment with RIT of LY294002 or U73122 prevented the cocaine-induced increase in DAF-2 fluorescence. Treatment with LY294002 alone also increased DAF-2 fluorescence, while the level of DAF-2 fluorescence observed following treatment with U73122 resembled that observed in RIT control cells, as shown in Figure 16. These data suggest that Akt and PKC, but not MAPK modulate the cocaine-induced increase in NO production.
Figure 15: Effect of cocaine and MAPK inhibitors on DAF-2 fluorescence. Representative phase contrast, fluorescence microscope images depicting DAF2 fluorescence in untreated PC12 cells (RIT control), and cells treated with 100 µM RIT cocaine or 50µM RIT U0126 alone or in combination. All cells were treated for 3 days (72 h).
Figure 16: Effect of cocaine and Akt and PKC pathway inhibitors on DAF-2 fluorescence. Phase contrast, DAF-2 fluorescence, and overlay of PC12 cells. Representative confocal microscope images depicting DAF-2 fluorescence in untreated PC12 cells in RIT control medium, cells treated with 100 µM RIT cocaine, cells treated with either 10µM RIT LY94002 or 5µM RIT U73122 inhibitor, and cocaine-treated cells grown in the presence of RITLY94002 or RIT U73122 inhibitor. All cells were treated for 3 days (72 h).
Effect of NOS inhibitor (L-NAME) and cocaine on Akt and MAPK activation levels in the PC 12 cell line

To determine whether NO modulated the cocaine-induced activation of signaling pathways, the effects of cocaine on Akt and MAPK activation was assessed. It was previously determined that inhibition of NOS activity using 20 mM L-NAME prevented the 100 µM *RIT* cocaine-induced increase in MOR protein and mRNA expression (Winick-Ng et al., 2012). Therefore, this dose and inhibitor was used in these studies.

Activation of Akt by cocaine

Figure 17A (upper panel) illustrates the levels of total Akt protein expression. No bands were detected using an antibody selective for phospho-Akt, but total Akt protein was assessed and the levels were analyzed densitometrically. In lysates obtained from PC12 cells treated with 100 µM cocaine for 30 min there did not appear to be activation of Akt in comparison to control samples. However, cocaine treated PC12 cells for 60 min and for 120 min showed an increase in total Akt levels. Tubulin levels (Figure 17A, lower panel) appear to be consistent across all treatments although the intensity of the bands was weak.

A summary of ratios for densitometric analysis of total Akt (normalized to tubulin) displayed as a function of cocaine treatment time were calculated to quantify total Akt protein levels in PC12 cells (Figure 17B). There were significant differences between treatments (**P<0.0001) with a significant and maximal increase in level of total Akt following 60 min and 120 min of exposure to cocaine (**P<0.001 for both), as shown in Figure 17B. Lysate treated with 30 min of cocaine
was not different from control, however it showed a statistical significance of *P<0.01. Akt activation in lysates obtained from cells pretreated with L-NAME prior to cocaine treatment, after 60 min and 120 min was significantly reduced compared to that observed following cocaine treatment alone, with P values of **P<0.01, **P<0.001 respectively. Akt activation in cells pretreated with L-NAME followed by 30 min cocaine exposure was not different from control or cocaine alone, as shown in Figure 17B.

A

**Total Akt**

![Image of Total Akt expression and tubulin blots with molecular weights](image1)

**α-tubulin**

![Image of α-tubulin blots with molecular weights](image2)

<table>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(+) L-NAME (20mM)</td>
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<td></td>
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</table>

B

![Bar chart showing relative total Akt activation](image3)

**Figure 17:** The effects of cocaine and L-NAME on total Akt activation in PC12 cells. (A) Total Akt expression (upper blot) and tubulin (lower blot) in extracts of
control cells or cells exposed to 100 µM RIT cocaine in the presence or absence of 20 mM L-NAME. (A) The lanes were loaded as follows: control, L-NAME, cocaine treated PC12 cells for 30 min L-NAME pretreated cells for 1 h then subjected to cocaine for 30 min cocaine treated PC12 cells for 60 min, L-NAME pretreated cells for 1 h then subjected to cocaine for 60 min, cocaine treated PC12 cells for 120 min, L-NAME pretreated cells for 1 h then subjected to cocaine for 120 min. Tubulin was used as a loading control. (B) Densitometric analysis of total Akt normalized to tubulin levels. Cocaine significantly increased total Akt activation following 30 min, 60 min, and 120 min of treatment (*P<0.01, *P<0.001, *P<0.001). The presence of L-NAME significantly decreased cocaine’s effect following 60 and 120 min of treatment **P<0.01 and **P<0.001 respectively, in comparison to cocaine alone. Statistical analysis was conducted using a one way ANOVA followed by Tukey test for significance between all pairs of columns. Results are shown as mean ± SEM. This figure is representative of immunoblots obtained from three independent experiments. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

Activation of MAPK by cocaine

Figure 18A (upper panel) illustrates the pattern of p44 (upper band) and p42 (lower band) MAPK phosphorylation following 100 µM cocaine in the presents and absence of 20 mM of L-NAME. Lysates obtained from untreated control PC12 cells, L-NAME treated cells, cocaine-treated cells for 30 min, and L-NAME pretreated cells prior to the treatment of cocaine for 30 min of exposure exhibited similar MAPK phosphorylation, but there was a rapid increase in phosphorylated MAPK levels with activation observed in samples obtained from cells exposed to cocaine for 60 and 120 min. However, cells exposed to cocaine for 120 min appeared to have less phosphorylation than cells treated for 60 min. L-NAME pre-treatment did not appear to affect MAPK phosphorylation at any of the treatment times.

Ratios for densitometric analysis of phospho-p42 and44 MAPK (normalized to tubulin) displayed as a function of cocaine treatment time were calculated to quantify the activation profile for p42 and p44 MAPK in the PC12 cell line (Figure 18B). Both p42 and p44 MAPK showed similar activation profiles. Lysates obtained from cells
treated with cocaine for 60 and 120 min showed an increased level of phosphorylation (*P<0.001 for both p42 and p44 MAPK), while 30 min of treatment had no effect. In the presence of L-NAME, MAPK activation in cells treated with cocaine for 60 and 120 min was also significantly elevated in comparison to control cells, and was not different from the level of MAPK activation observed in cells treated with cocaine alone.

The only difference between the p44 MAPK and p42 MAPK activation profiles is that phosphorylation of p44 MAPK in L-NAME pretreated cells prior to 30 min cocaine exposure was significantly higher than control and cocaine 30 alone (*P<0.05 for both), while p42 MAPK activation was not different, as shown in Figure 18C. These data indicated that cocaine-mediated increases in NO production do not modulate MAPK activation in PC12 cells.

A

**p-MAPK**

<table>
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<td>+</td>
<td>+</td>
</tr>
<tr>
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**α-tubulin**

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<tr>
<td>(+)L-NAME(20mM)</td>
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**Figure 18:** Temporal pattern for phosphorylation of p44 and p42 MAPK in cocaine-treated PC 12 cells with the presents and absence of L-NAME. (A) Representative immunoblots of phosphorylation of p44 (upper band) and p42 (lower band) MAPK, protein lysates from control (untreated) in the first lane followed by 20 mM of L-NAME-treated cells, and cocaine (100 µM) treated PC 12 cells for 30 min, then 60 min, then 120 min in the presents and absence of 20 mM L-NAME on the nitrocellulose membranes. Phosphorylated forms of MAPK were detected by incubating membranes with phosphorylation-specific MAPK antibody. Maximal phosphorylation of p42 and p44 MAPK appeared to occur after 60 min of cocaine treatment with the gradual decrease in activation by 120 min of cocaine treatment. (B) Densitometric analysis of p42 MAPK normalized to tubulin levels. Following both 60 and 120 min cocaine treatment p42 MAP kinase was significantly activated in comparison to control (*P<0.001). Both L-NAME pretreated cells prior to cocaine
treatment for 60 and 120 min showed also a significant activation profile in comparison to control lysate (*P<0.001 for both), and this activation was not different from cocaine treatment alone. Akt phosphorylation following treatment with cocaine for 30 min in the presence or absence of L-NAME was not different from control. (C) Densitometric analysis of p44 MAPK normalized to tubulin levels. Phosphorylation of p44 MAPK was significantly higher than control in extracts obtained from cells treated with cocaine for 60 and 120 min in both the absence and presence of cocaine. Treatment with cocaine alone for 30 min did not increase MAPK phosphorylation but in combination with L-NAME, 30 min cocaine treatment resulted in a significant increase in p44 MAPK activation in comparison to control and cocaine alone(*P<0.05 for both). Statistical analysis was conducted using a one way ANOVA followed by Tukey test for significance between all pairs of columns. Results are shown as mean ± SEM. This figure is representative of immunoblots obtained from three independent experiments. One asterisk symbol (*) verifies statistical significance to control levels, two asterisk symbols (**) verifies statistical significance to cocaine treatment levels, three asterisk symbols (***) verifies statistical significance to inhibitor levels.

**DISCUSSION AND CONCLUSIONS**

Previous studies indicated that cocaine can indirectly modulate a number of neurochemical systems, including the endogenous opioid system (Cummins et al., 2009; Roth-Deri et al., 2008; Shippenberg et al., 2007), and some have indicated that cocaine increases MOR mRNA and peptide expression (Winick-Ng et al., 2012; Bailey et al., 2005; Azaryan et al., 1996) in regions of the brain known to regulate incentive motivation and stress reactivity (Koob et al., 2007; Di Chiara et al., 1995). The mechanisms involved in this regulation require a clear understanding of the cocaine-MOR signaling pathways. In the present study these pathways were examined in the PC12 cell line. Since the activation of signaling pathways in these cells following cocaine treatment had not been characterized previously, the activation profiles of these pathways were investigated under the influence of cocaine treatments and modulation of these pathways by NO also was examined. The present study demonstrated for the first time (1.) the characterization of cocaine mediated signaling pathways in the PC12 cell line; the effect of different pathway inhibitors on PC12 cells, the PLC-PKC inhibitors U73122 and BIS-1 were used to investigate the
involvement of the PKC signaling pathways in MOR expression levels, the evaluation of MAPK pathway by the use of U0126 inhibitor, and the LY94002 inhibitor was used to investigate the PI3K/Akt pathway; (2.) the assessment of NO production levels via different pathways which are activated during RIT of cocaine, and (3.) the modulation of MAPK and Akt pathways activation by the non-selective NOS inhibitor L-NAME in cocaine-treated PC12 cells. Moreover, the present study identified a number of cellular mechanisms by which cocaine alters MOR expression; it was shown also for the first time that different cocaine treatment regimens; treatment of PC12 cells with either a single dose (SCT) or repeated doses of cocaine (RIT) increased MOR protein levels via different signaling pathways. Therefore, it is concluded that mechanisms regulating increases in MOR expression levels were dependent on the treatment regimen used.

In this study the effects of cocaine on MOR protein levels were evaluated in extracts of control and treated PC12 cells using western blot analysis. Denitometric analysis of immunoblots depicting MOR levels and the corresponding α-tubulin levels obtained from cells treated with 500 and 100 μM SCT and RIT cocaine for 72 h revealed a significant increase in MOR protein levels relative to control in PC12 cells. This data supports the study done by Winick-Ng et al., 2012.

Moreover, the effects of cocaine induced MOR up-regulation expression levels on transcription were also assessed. PC 12 cells were treated with actinomycin D in order to study the effects cocaine has on transcription and subsequently on MOR protein expression levels. Winick-Ng et al. studied the effects of cocaine on MOR via measuring mRNA levels; our results complimented this study in measuring protein
levels in cocaine-induced MOR. When RNA was isolated from cells treated for 48 h with a SCT 500 μM dose, or RIT 100 μM doses of cocaine followed by real time PCR (qPCR) analysis, a significant increase in relative MOR mRNA levels was detected in cells treated with 100 μM RIT cocaine for 48h compared to control, and there was no change in MOR mRNA expression following 500 μM of SCT cocaine (Winick-Ng et al., 2012). This effect of dosing regimen is in agreement with previous reports describing differences in behavioral outcomes associated with the MOR, including enhanced sensitization due to repeated cocaine exposure (Caster et al., 2005; Davidson et al., 2002; Unterwald et al., 2001). This study explored the levels of MOR protein expression mediated by transcription that were determined by western blot analysis. Densitometric data revealed that actinomycin D significantly reduced the levels of MOR protein expression after RIT 100 μM of cocaine. Therefore, it is concluded that cocaine, in fact, does regulate MOR expression by increasing protein levels at both transcriptional (RIT) and post-transcriptional (SCT) levels.

Also, the present study investigated the activation profiles of cocaine signaling pathways that were characterized in the PC12 cell line. The first investigation was the phospholipase C -protein kinase C (PKC) signaling pathway. This pathway is very complicated as there are at least 12 PKC isoforms and possible cross talk with the MAPK pathway. Protein Kinase C is a family of serine/threonine kinases that fall into 3 main classes: conventional (α, βI, βII, γ), novel (δ, ε, θ, and μ) and atypical (ζ, λ, and ι). The identity of the PKC isoforms that are expressed in the PC12 cell line has not been elucidated in this study. The PLC-PKC inhibitors U-73122 and BIS-1 were used to investigate the involvement of the PKC signaling pathways in MOR expression levels. BIS-1 shows a high selectivity for PKC-α, βI, βII, γ, δ, and ε
Isozymes, while U73122 is an inhibitor of phospholipase C, an upstream activator of PKC and other molecules. SCT BIS-1 significantly decreased cocaine-induced MOR protein up-regulation expression levels, however, RIT treatment regimen for BIS-1 inhibitor partially decreased MOR protein levels, but it was considered a non-significant decrease. Also, SCT U73122 inhibitor significantly reduced cocaine-induced MOR protein expression levels to control levels. Still, RIT U73122 did reduce cocaine-induced MOR protein expression levels, but the difference was not significant according to GraphPad statistical analysis. Onaivi et al. (1998) reported that cocaine disrupts signal transduction in PC 12 cells by altering the expression and activity of PKC isoforms and Ca2+ levels in vitro. This study compliments the findings as cocaine did in fact up-regulate the MOR expression level through the PKC pathway in SCT of cocaine. It is concluded that there were significant differences between treatments, treatment regimens, and mechanisms regulating these increases in MOR expression levels.

There is crosstalk between the PKC pathway and the MAPK pathway (Almela et al., 2009) although, these differences in mechanisms can be either cross talk between the PKC pathway and the MAPK pathway; one or more of the PKC isoforms is just an upstream activator of the MAPK pathway or simply that different dosage regimens of cocaine have different mechanisms of actions resulting in differences in gene regulation. These results could be more clarified by further experiments.

The effects of U0126 and LY294002 inhibitors for the MAPK and PI3K/Akt pathways, respectively on cocaine-induced MOR protein up-regulation expression levels were also assessed. MEK1 and MEK2, also called MAPK or Erk kinases, are dual-specificity protein kinases that function in a mitogen activated protein kinase
cascade controlling cell growth and differentiation in PC12 cells (Rosen et al., 1994; Resing et al., 1995; Crews et al., 1992). U0126 is a highly selective inhibitor of both MEK1 and MEK2 (MAPK/ERK kinase) and in the present study, U0126 prevented the cocaine-mediated increase in MOR levels following both SCT and RIT. Studies have demonstrated that blockade of ERK phosphorylation in vivo in the NAc by single bilateral microinjections of PD98059 inhibitor, or U0126 into this site, inhibited the expression of cocaine-induced behavioral sensitization (Kim et al., 2011). This in vivo study supported the results obtained in this thesis. These investigations were further extended in this thesis in vitro; studying the cocaine-induced up-regulation of MOR expression levels in the PC12 cells with different dosage regimens. Moreover, the PI3 Kinase-Akt pathway has been shown to have a major role in cell survival and overexpression of Akt in primary cultures of sympathetic and cerebellar neurons provides protection against cell death due to PI3 inhibition or serum starvation (as reviewed in Sofroniew, 2001). Other studies indicated cocaine effects on ERK/Akt phosphorylation were inhibited by pre-incubation with the phosphatidylinositol 3-kinase inhibitor LY294002, suggesting that MAPK and Akt pathways are critical mediators of cocaine-induced structural plasticity in vivo, suggesting their involvement in cocaine addiction (Collo et al., 2012). SCT and RIT treatment regimens were explored for both pathways. Both RITs of cocaine complimented the SCTs of cocaine by significantly reducing cocaine-induced MOR protein expression levels to control levels. These results suggested that MAPK and Akt are activated in both treatment regimens, and that they are the pathways activated in cocaine-induced up-regulation of MOR in PC12 cells. Whether they are activated in a parallel/individual manner or in a sequential manner should be the focus of additional investigations.
The free radical nitric oxide (NO) is produced during the conversion of arginine to citrulline by the class of enzymes known as nitric oxide synthases (NOS) (Mayer et al., 1989). There are 3 NOS isoforms present in the brain, the neuronal and endothelial isoforms (nNOS and eNOS respectively) are constitutively expressed and there is also an inducible isoform (iNOS). The present study investigated the relationship of NO in regulating cocaine-induced changes in MOR protein levels and the effects of the different pathways that NO maybe linked to. Previous studies have linked NO to cocaine-induced modulation of MOR (Winick-Ng et al., 2012), but to study NO effects on the signaling pathways, are novel findings. In PC12 cells, pretreatment with the non-selective NOS inhibitor L-NAME prevented cocaine-induced up-regulation of MOR mRNA and protein (Winick-Ng et al., 2012). Additionally, NO production was assessed visually and both 100 μM RIT and 500 μM SCT cocaine substantially enhanced DAF-2 fluorescence relative to control (untreated) cells indicating both dosing regimens result in increased NO production (Winick-Ng et al., 2012). Interestingly, in in vivo neuronal models, cocaine increases NO production by increasing neuronal NOS (nNOS) protein levels and activation (Sammut et al., 2008) via dopamine-, glutamate- and MOR-dependent mechanisms (Yoo et al., 2006). The specific target of cocaine in altering NO levels or activity yet remains unknown. In this study, DAF-2 fluorescence appeared to be increased in cells treated with 100 μM RIT cocaine. Treatment with U0126 alone had no effect on DAF-2 fluorescence and when cells were pretreated with U0126, cocaine was still able to increase DAF-2 fluorescence. In contrast, pretreatment with RIT of LY294002 or U73122 prevented the cocaine-induced increase in DAF-2 fluorescence. Treatment with LY294002 alone also increased DAF-2 fluorescence, while the level of DAF-2 fluorescence observed
following treatment with U73122 resembled that observed in RIT control cells. In conclusion, MAPK may be activated in cocaine-induced MOR up-regulation in an individual manner when NO is also activated, but MAPK does not play a role in the cocaine-induced increase in NO production, suggesting they do not interact with one another. On the other hand, Akt pathway activation seems to affect NO production levels, since the inhibitor prevented NO production. Moreover, the PKC pathway inhibitor, U73122 also prevented NO production levels in cocaine treated PC12 cells. These results confirmed that PKC and Akt are pathways activated in cocaine-treated PC12 cells and that these pathways interact with NO in up-regulating the MOR protein expression. However, MAPK does not modulate the cocaine-induced increase in NO production. Further investigation via quantitative measures may be required for reassurance of these outcomes, as this investigation was simply qualitative.

Finally, in this study, the activation profiles for cocaine signaling pathways were characterized in the PC12 cell line depending on the DAF-2DA fluorescence results. As studies provided evidence that the activation profile of phosphor-Akt in different regions in the rat's brain in vivo, and the Akt pathway is impacted by RIT of cocaine exposure (Perrine et al., 2008). Total Akt was assessed after the treatment of the PC12 cells with cocaine with or without the non-selective NO inhibitor L-NAME. Total Akt levels were assessed densitometrically following the treatment of PC12 cells with 100μM cocaine. In lysates obtained from PC12 cells treated with 100 μM cocaine for 30 min did not increase total protein levels of Akt in comparison to control samples. However, cocaine treated PC12 cells for 60 and 120 min showed an increase in total Akt protein levels. Also, total Akt protein levels in lysates obtained from cells pretreated with L-NAME prior to cocaine treatment, after 60 and 120 min was
significantly reduced compared to that observed following cocaine treatment alone. Concluding that NO modulates cocaine-induced total Akt protein levels. The activation profile of Akt must be assessed by further experiments using the phosphor-Akt antibody. These were primary experiments showing overall total Akt protein expression levels, and it seems that NO is involved. Further experiments will give a clarified view.

Moreover, the activation of p44 and p42 MAPK phosphorylation following 100µM RIT cocaine in the presence and absence of 20 mM of L-NAME was assessed. Studies have linked the MAPK pathway with cocaine in in vivo studies (Kim et al., 2011; Chen et al., 2010). However, the link of NO to the MAPK pathway in cocaine-induced PC12 cell line remains unknown. This study demonstrated novel findings on the activity profile of MAPK after the inhibition of the cocaine-induced up-regulation expression by the non-selective inhibitor L-NAME. This experiment was also performed to complement the findings of the DAF-2 fluorescence experiments. Lysates obtained from cocaine-treated cells for 30 min, and L-NAME pretreated cells prior to the treatment of cocaine for 30 min of exposure exhibited similar MAPK phosphorylation levels, but there was a rapid increase in phosphorylated MAPK levels with activation observed in samples obtained from cells exposed to cocaine for 60 and 120 min. However, L-NAME pretreatment did not appear to affect MAPK phosphorylation at any of the treatment time points. Both p42 and p44 MAPK showed similar activation profiles. Thus, these results indicate that similar to the DAF-2 fluorescence experiments, following cocaine treatment, NO and MAPK do not interact. In conclusion, data indicated that cocaine-mediated increases in NO production do not modulate MAPK activation in PC12 cells.
Limitations and Future Directions

These novel findings could have significant impact on the future of cocaine relapse therapy investigation and highlight the importance of the cellular models used to investigate gene regulation mechanisms in normal and disease states. With so many previous studies using the PC12 cell line, it is important to know whether this model is representative of both neurons and human brain cells. By understanding the limitations of cell model systems and how these cells may differ, we may be better able to determine how best to interpret data from these studies that will contribute in developing further research and treatment options for cocaine relapse. PC12 cells are rat pheochromocytoma cell line that has been well characterized and used in many previous studies as a representative model for a neuronal cell. However, the use of human derived cell lines may provide a better understanding of the cellular mechanisms that cocaine may exert on the MOR i.e. the SH-SY5Y cell line; human derived cell line isolated from a bone marrow biopsy taken from a four year-old female with neuroblastoma, or the IMR32 cell line which is also a neuroblastoma cell line. Attempts were made to detect MOR levels in these cell lines, regrettfully I was not able to obtain reliable results from the human derived cell lines that can be reported here and therefore these cells were not assessed in mechanisms of cocaine up-regulating the MOR. Also, in contrast to neurons, PC12 cells continue to divide which could make them less vulnerable to neurotoxic compounds. Although we have identified many similarities between our findings and previous in vivo studies, differences in the biology of cocaine in the intact organisms compared to the effects observed in vitro are likely to exist.
In addition, for future studies in the PC12 cell line, the promoter of the MOR could be cloned and attached to luciferase. Therefore, the promoter will be activated, and more luciferase will get to be made, facilitating quantitative measurement of MOR transcriptional activation. Also, in order to assess the mechanism model presented in Figure 5, a dopamine antagonist can be administered prior to treatment of cocaine, for both dosage regimens, also testing the PLC-PKC pathway that might be involved. As recent studies suggested that the D₁ dopamine receptor (D₁R) has been proposed to form a hetero-oligomer with the D₂ dopamine receptor (D₂R), which in turn resulted in a complex that coupled to PLC-mediated intracellular calcium release (Chun et al., 2013). The study concluded that the mechanism of D₁R-D₂R-mediated calcium signaling involved more than receptor-mediated G protein activation, and it may largely involve downstream signaling pathways, which could be related to the PKC pathway. Also, that there is significant cross-reactivity to other receptors, suggesting the possibility of the MOR being involved, as proposed in Figure 20. Also, the relationship of the PLC-PKC pathway to NO is yet to be investigated.

Also, in order to complete the experiments proposed in this study the NO production for the SCT is yet to be explored, as proposed in Figure 19. In addition, the mRNA levels of MOR were not explored in this study, although there is an overall understanding of the mechanisms involved in the cocaine-induced increase in MOR protein levels by western blotting. The RNA data performed by PCR will confirm the results and findings in this study, in the same order as was proposed previously in Figure 6. Moreover, the phospho-Akt activation levels could be studied further, in order to ensure that L-NAME affected the activation of this pathway in particular. As for the DAF-2 fluorescence assay, this assay could be quantified rather than depending
on qualified data in measuring NO production levels due to cocaine exposure, as
presented in this study.

**Figure 19:** Schematic representation of *SCT* cocaine's action within the PC12 cells. The three pathways: PLC-PKC, MAPK, and PI3K-Akt were all activated during *SCT* of cocaine in regulating the cell. Although, NO production's relationship to each pathway during *SCT* of cocaine was not assessed.
Figure 20: Schematic representation of RIT cocaine’s action within the PC12 cells. Only two pathways: MAPK, and the PI3K-Akt were activated during RIT of cocaine in regulating the cell. NO production have been found to be regulated via the PLC-PKC pathway, and the PI3K-Akt pathway, but not by the MAPK pathway. The PLC-PKC pathway is yet to be explored in relation to the dopamine receptors in up-regulating the MOR via NO.

Summary and Conclusions

Cocaine effects and the signaling pathways involved in the up-regulation of MOR in the PC12 cell line was studied by using two treatment regimens; SCT and RIT. SCT of cocaine in PC12 cells seem to have all three pathways activated in MOR expression levels; PKC, MAPK, and the Akt pathway. However, RIT of cocaine only activated the MAPK and Akt pathway, suggesting that different treatment regimens of cocaine activate different pathways in cocaine up-regulating the MOR. Also, NO seem to be involved in cocaine-induced up-regulation MOR in PC12 cells. NO seemed to be involved with the PKC and Akt pathways in up-regulating MOR in RIT of cocaine. On the other hand, NO and MAPK modulate the MOR up-regulation
simultaneously, but in an individual/parallel manner. Results showed that NO appeared to modulate the PKC and Akt pathways in the cocaine-mediated up-regulation of the MOR expression directly by the Akt pathway, and indirectly by the PKC pathway. Depending on DAF-2 fluorescence experiments, activation levels of both Akt and MAPK were investigated via NO. Results showed that NO activated levels of the Akt pathway, but did not affect the MAPK level of activation. Thus, findings indicated that Akt and NO act together in up-regulating the MOR, but MAPK acts on the MOR in an individual manner. In conclusion, different treatment regimens of cocaine activate different pathways; SCT of cocaine activated all three signaling pathways, however, RIT of cocaine activated the MAPK and Akt pathways.
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