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THE MESOCORTICOLIMBIC DOPAMINE PATHWAY RECONSTITUTED IN
VITRO: GLUTAMATE RECEPTORS AND CORTICOSTEROID-
METHAMPHETAMINE NEUROTOXICITY

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Science at the University of Kentucky

By

Jennifer Nicole Berry

Lexington, Kentucky

Director: Dr. Mark Prendergast, Professor of Psychology
Lexington, Kentucky

2013

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ABSTRACT OF DISSERTATION

THE MESOCORTICOLIMBIC DOPAMINE PATHWAY RECONSTITUTED IN VITRO: GLUTAMATE RECEPTORS AND CORTICOSTEROID-METHAMPHETAMINE NEUROTOXICITY

Stress promotes the use of methamphetamine and other recreational substances and is often implicated in relapse to stimulant use. Thus, it is of critical importance to examine the consequences of the co-occurrence of stress and methamphetamine use. Activity of the glutamatergic *N*-methyl *D*-aspartate (NMDA) receptor system appears to be involved in the neurotoxic effects of both chronic stress and methamphetamine exposure. The current studies investigated the hypothesis that chronic pre-exposure to the stress hormone corticosterone (CORT) results in an increase of NMDA receptor activity and that this will potentiate the neurotoxic effects of methamphetamine (METH). Co-cultures of the ventral tegmental area, nucleus accumbens, and medial prefrontal cortex were pre-exposed to CORT (1 μ M) for 5 days prior to co-exposure to METH (100 μ M) for 24 hours to investigate the combined effects on neurotoxicity and protein density of NMDA receptor subunits. The combination of CORT and METH resulted in significant neurotoxicity within the medial prefrontal cortex compared to either CORT or METH alone. The CORT+METH-induced toxicity was attenuated by co-exposure to the NMDA receptor antagonist (*2R*)-amino-5-phosphonovaleric acid (APV; 50 μ M) during the 24 hour CORT and METH co-exposure. Although CORT alone did not significantly alter the density of the NR1 and NR2B subunits of the NMDA receptor, METH exposure for 24 hours resulted in a significant loss of the polyamine sensitive NR2B subunit. Co-exposure to CORT and METH also resulted in decreased extracellular glutamate while not significantly altering extracellular dopamine. These results suggest an enhancement of NMDA receptor systems or downstream effectors in areas of the mesolimbic reward pathway following chronic pre-exposure to CORT, which leads to enhanced neuronal vulnerability to future excitotoxic insults. This may be of critical importance as use of

psychostimulants such as METH and other drugs of abuse may produce excitotoxic events in these areas, thus further compromising neuronal viability.

KEYWORDS: Corticosterone, methamphetamine, NMDA receptor, co-culture, mesolimbic dopamine system

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Chapter 1

INTRODUCTION

Stress and Substance Abuse

Psychological and physical stressors often precede and accompany substance abuse as well as precipitate relapse. Individuals with a history of substance abuse often report stress and negative mood states as reasons for relapse and continued abuse of substances (Jacobsen, Southwick, & Kosten, 2001). High rates of co-morbidity between post-traumatic stress disorder (PTSD) and substance abuse disorders were first reported in war-related studies; in these investigations, as many as 75% of combat veterans diagnosed with lifetime PTSD also reported substance abuse or dependence (Jacobsen, et al., 2001). Furthermore, an earlier study revealed that adolescents who reported physical and sexual abuse were more likely to use alcohol and marijuana, were more likely to abuse multiple substances, and reported earlier initiation of drug use compared to their non-abused counterparts (Harrison, Fulkerson, & Beebe, 1997). A longitudinal study conducted with young adults found that those diagnosed with PTSD were nearly five times more likely to develop drug abuse or dependence during the 12-month assessment interval than those experiencing no trauma (Reed, Anthony, & Breslau, 2007). Indeed, several studies have noted increased use of psychostimulants such as cocaine and amphetamine in individuals diagnosed with PTSD or anxiety disorders (Sareen, Chartier, Paulus, & Stein, 2006; Solomon, Kiang, Halkitis, Moeller, & Pappas, 2010). A recent study investigating the use of crystal methamphetamine found that individuals diagnosed with PTSD were significantly more likely to report previous crystal methamphetamine use, began use at an earlier age, and use crystal methamphetamine over five times longer

than their trauma-exposed, non-PTSD counterparts (Smith, Blumenthal, Badour, & Feldner, 2010). Further, acute administration of the stress hormone cortisol elicited increased subjective ratings for craving in cocaine-dependent individuals (Elman, Lukas, Karlsgodt, Gasic, & Breiter, 2003). In addition, images of personal stressors increased ratings of anxiety and drug craving in cocaine-dependent individuals, abstinent alcoholics, and in naltrexone-treated opiate-dependent individuals (Sinha, 2007). Collectively, these studies demonstrate the high rate of co-morbidity between stress disorders such as PTSD and substance abuse.

Preclinical research with animal models lends further support for the link between stress and substance abuse. Rodent models employing intermittent stressors, such as footshock or forced swim periods, have been shown to produce cross-sensitization to many psychostimulant drugs and enhance the rewarding properties associated with these drugs of abuse (as reviewed in Yap & Miczek, 2008). Additionally, exposure to physical stressors has been shown to facilitate the acquisition of psychostimulant self-administration (Kosten, Miserendino, & Kehoe, 2000; Piazza, Deminiere, le Moal, & Simon, 1990). Additionally, exposure to an acute stressor (i.e. footshock) can induce reinstatement of drug-seeking behavior for many drugs of abuse (for reviews, see Cleck & Blendy, 2008; Yap & Miczek, 2008). Further evidence of the interaction between stress and psychostimulant intake in preclinical models is often found in studies involving enriched (i.e. social cagemates and novel toys) versus isolated rearing conditions. Several studies have noted increased amphetamine self-administration and increased amphetamine-induced locomotor sensitization in rats raised under isolated conditions as opposed to enriched conditions (Bardo et al., 1995; Bardo, Klebaur, Valone, & Deaton,

2001; for a review, see Stairs & Bardo, 2009). Additional studies have suggested that the increased amphetamine self-administration observed in socially isolated rodents may be mediated by stress and the glucocorticoid receptor (Stairs, Prendergast, & Bardo, 2011). Taken together, both human and rodent studies suggest that high levels of stress may play a role both in the initiation of substance use and in relapse.

Mesocorticolimbic Dopamine Reward Pathway

The primary reinforcing effects of all drugs of abuse, as well as all natural reinforcers (i.e. food, water, sex), are thought to be dependent, at least in part, on the mesocorticolimbic dopaminergic reward pathway. This pathway consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc), which is subdivided into the core and shell. Broadly speaking, this pathway also contains glutamatergic and GABAergic fibers that project from limbic structures such as the hippocampus, amygdala, and medial prefrontal cortex (mPFC) to the NAcc as well as dopaminergic tracts that project from the VTA to the aforementioned limbic structures (for a review, see Fields, Hjelmstad, Margolis, & Nicola, 2007; Pierce & Kumaresan, 2006). Although the VTA contains both GABAergic and glutamatergic neurons that project to the NAcc and other limbic regions, it is the neurotransmitter dopamine (DA) and the dopaminergic projections to the NAcc which have been the focus of many studies investigating the rewarding effects of drugs of abuse. Early studies revealed that direct stimulation of the VTA or NAcc is rewarding such that these areas sustain intra-cranial self-stimulation, ICSS (Fibiger & Phillips, 1988; Robbins & Everitt, 1996). Activation of the mesocorticolimbic pathway leads to increased DA levels most often in the NAcc, but also in the prefrontal cortex (PFC). It is this increase in DA which

is thought to be responsible for the rewarding and pleasurable feelings associated with a stimulus (Addolorato, Leggio, Abenavoli, & Gasbarrini, 2005). In sum, the mesocorticolimbic pathway and resultant rise of DA in the NAcc appear to be critical for all reinforcers.

Glutamate receptors: Structure and Function

Glutamate (GLUT) is the major excitatory neurotransmitter in the mammalian brain and activates both ionotropic and metabotropic receptors. While the metabotropic receptors produce actions via G-proteins (linked to these receptors) to possibly release intracellular stores of calcium (Ca^{2+}), ionotropic receptors act via ion channels to allow Ca^{2+} , sodium (Na^+), and/or potassium (K^+) to flow into the cell. The glutamatergic ionotropic family of receptors includes the kainate receptor, the 2-amino-3(3-hydroxy-5-methylisoxazol-4-yl) propanoate (AMPA) receptor, and the *N*-methyl-*D*-aspartate (NMDA) receptor.

AMPA receptors are heteromeric structures composed of four different subunits, GluR1-4, which usually combine to form heterotetramers. Each subunit consists of a membrane region with three transmembrane domains (TM1, 3, and 4) and a second domain which loops back within the membrane and comes out the intracellular side (M2). Thus, each subunit has an extracellular N-terminal domain and an intracellular C-terminal domain. Of the four subunits, it is only the GluR2 subunit that can undergo RNA editing in the second membrane to produce a positively charged arginine (R) residue as opposed to the neutral glutamine (Q) residue that is present on unedited GluR2 subunits (Jonas & Burnashev, 1995). This editing of the GluR2 subunit mediates the receptor's

permeability to Ca^{2+} and other ions (Jonas & Burnashev, 1995). In particular, receptors missing the GluR2 subunit exhibit a high permeability to Ca^{2+} and other cations, while receptors containing the edited GluR2, and therefore the arginine residue, are impermeable to Ca^{2+} (Jonas & Burnashev, 1995). Previously it was thought that all AMPA receptors were impermeable to Ca^{2+} ; however, it is now known that this is not the case as AMPA receptor antagonists have been shown to be neuroprotective against Ca^{2+} -induced excitotoxicity (for a review, see Gill & Lodge, 1997). The AMPA receptor is blocked by polyamines under resting conditions and is only opened when the membrane becomes depolarized to reach the threshold for an action potential. Once the polyamine blockade is removed, the ion channel opens to allow Na^+ (and Ca^{2+} , if applicable) into the cell. This further depolarizes the cell and can lead to activation of the NMDA receptor, as AMPA and NMDA receptors are often co-localized in the cell membrane. This close proximity between AMPA and NMDA receptors is important in memory processes such as long-term potentiation (LTP) and long-term depression (LTD; for a review, see Wheal et al., 1998).

The kainate receptors, although not yet well characterized, are thought to be made up of tetramers comprised of various subunits including GluR5-7 and KA1-2 (for a review, see Heath & Shaw, 2002). Structurally, each subunit is thought to be homologous to the subunits of the AMPA receptor (Sattler & Tymianski, 2001). The GluR5-7 subunits have a relatively low affinity for kainate as compared to the high-affinity KA1 and KA2 subunits (reviewed in Lodge, 2009). The high-affinity KA1 and KA2 receptor subunits cannot form functional receptors alone but can combine with GluR5 or GluR6 to form functional receptors (Lodge, 2009). Similar to the GluR2

subunit of the AMPA receptor, GluR5 and GluR6 can undergo RNA (Q/R) editing in transmembrane domain 2; this editing is thought to mediate the channel's permeability to Ca^{2+} (Pinheiro & Mulle, 2006). The kainate receptors have been shown to both facilitate and inhibit the synaptic release of GLUT when kainate receptors are found on the presynaptic nerve cell and thus serve as autoreceptors (see Jane, Lodge, & Collingridge, 2009 for a review).

NMDA receptors are ionotropic glutamatergic receptors which are tetramers made up of two essential NR1 subunits and two regulatory subunits composed of NR2A-D and/or NR3A-B subunits, although NR2A and NR2B are the most commonly expressed regulatory subunits (for a review, see Cull-Candy, Brickley, & Farrant, 2001). Structurally, the subunits are similar to AMPA receptor subunits, each possessing four membrane domains (3 transmembrane-spanning domains and one membrane domain that loops back around), an extracellular N-terminal domain, and an intracellular C-terminal domain (as reviewed in Paoletti & Neyton, 2007). The ion channel is blocked (voltage-dependently) by Mg^{2+} , which is removed via membrane depolarization to allow Ca^{2+} and a small amount of Na^+ into the cell (Lynch & Guttman, 2001). As noted earlier, AMPA receptor depolarization is a common mechanism by which the Mg^{2+} blockade is removed. Glycine serves as a co-agonist at the NMDA receptor and must be bound to the NR1 subunit before the receptor is fully activated; however, the GLUT binding site is located on the NR2 subunit and must also be bound (Paoletti & Neyton, 2007). Thus, the opening of the NMDA receptor ion channel requires three distinct events: removal of the Mg^{2+} blockade, glycine binding, and GLUT binding (Lynch & Guttman, 2001). Polyamines, protons, and extracellular Zn^{2+} also act allosterically to modify receptor

selectivity (Cull-Candy, et al., 2001). NMDA receptor antagonists, such as MK-801, ketamine, and phencyclidine (PCP), are ion channel blockers which are non-competitive with NMDA (Lynch & Guttman, 2001).

While the NR2 and NR3 subunits are regulatory, the NR1 subunit is essential for functional NMDA receptors. Genetic deletions of the NR1 subunit have proven to be lethal within the early postnatal period (Forrest et al., 1994). In contrast, deletion of the NR2A subunit in mice resulted in survival and normal developmental appearance (Sakimura et al., 1995). Previous research has also shown the NR1 subunit to be essential for immediate GLUT- and NMDA-mediated toxicity (Tokita et al., 1996). Alongside the various sensitivities of the NR1 receptor, each NR2 subunit also has distinctive sensitivities which give rise to the many combinations of various receptor subtypes. Specifically, the NR2 receptor subunits each exhibit different sensitivities to the Mg^{2+} block, polyamines, glycine, and GLUT affinity (Kutsuwada et al., 1992; Monyer et al., 1992; Sharma & Reynolds, 1999; Stern, Behe, Schoepfer, & Colquhoun, 1992). The NR1 subunit has a total of eight functional splice variants, each of which has a different sensitivity to agonist, antagonists, Ca^{2+} and other ions, as well as one nonfunctional splice variant (see Zukin & Bennett, 1995 for a review). An asparagine residue (N598) found on the second membrane domain on both NR1 and NR2 subunits regulates the permeability to Ca^{2+} , Mg^{2+} , Na^+ , polyamines, and Zn^{2+} , with contrasting effects on the NR1 and the NR2 subunit (Burnashev et al., 1992; Schneggenburger & Ascher, 1997; Traynelis, Burgess, Zheng, Lyuboslavsky, & Powers, 1998). Specifically, replacing the asparagine residue with a glutamine residue on the NR1 was found to slightly reduce the Mg^{2+} blockade and decrease Ca^{2+} permeability, while the same

replacement on the NR2 did not significantly affect Ca^{2+} permeability but did increase Mg^{2+} permeability and more strongly reduced the Mg^{2+} blockade (Burnashev, et al., 1992).

Each NMDA receptor subunit demonstrates differences in their intracellular C-terminal domain sequence, which is thought to mediate the interaction of the NMDA receptor and various intracellular cytoskeletal and synaptic proteins (for reviews, see Arundine & Tymianski, 2004; Forder & Tymianski, 2009). The NMDA receptor and related proteins make up a large multiprotein structure located beneath the postsynaptic membrane known as the postsynaptic density (PSD). Postsynaptic density proteins are involved in various functions, including regulation of receptor clustering, modulation of receptor function, and cell to cell bonding (Forder & Tymianski, 2009). Moreover, there are four key types of molecules within the PSD including cytoskeletal, membrane-bound, modulatory enzymes, and scaffolding proteins (Forder & Tymianski, 2009). The interactions between the NMDA receptor and PSD proteins are highly specific, indicating that these interactions connect the NMDA receptor to particular downstream signaling molecules (Arundine & Tymianski, 2004).

Dopamine and Glutamate Interactions

There is also evidence of interactions between dopaminergic and glutamatergic neurons within the mesocorticolimbic pathway. Although this pathway is primarily thought to be DA-mediated, fewer than 60% of VTA neurons in the rat brain are dopaminergic (Margolis, Lock, Hjelmstad, & Fields, 2006). In addition to dopaminergic projections, the VTA is also thought to contain GABAergic and glutamatergic neurons

projecting to the NAcc, PFC, and other structures (Carr & Sesack, 2000; Chuhma et al., 2004; Kawano et al., 2006; Mendez et al., 2008; Van Bockstaele & Pickel, 1995). Additionally, a recent study found that the PFC and NAcc receive GLUT-only and GLUT-DA inputs from the VTA, paralleling the dopaminergic tracts (Yamaguchi, Wang, Li, Ng, & Morales, 2011). One theory that has been postulated in recent years is that GLUT is a co-transmitter in DA-releasing neurons (Fields, et al., 2007). Consistent with this theory, studies have found that activation of VTA neurons can produce glutamatergic excitatory postsynaptic potentials in medial spiny neurons of the NAcc, indicating that DA and GLUT may be co-released (Chuhma, et al., 2004; Hnasko et al.; Stuber, Hnasko, Britt, Edwards, & Bonci, 2010). One recent study found that GLUT co-release promotes the growth and survival of mesencephalic DA neurons in mice, as evidenced by a reduction in the density of DA innervation to the ventral striatum as well as a decrease in basal extracellular DA levels in mice with a conditioned deletion of the vGLUT2 gene in DA neurons (Fortin et al., 2012). Further, the DA-GLUT interaction may depend on the NMDA receptor, as stimulation of prefrontal glutamatergic NMDA receptors dose-dependently reduced basal and stimulated DA release in the PFC (Del Arco & Mora, 2001), whereas blockade of the NMDA receptors increased levels of basal DA and DA metabolites in the NAcc (Del Arco, Segovia, & Mora, 2008). Additionally, chronic antagonism of DA neurons *in vitro* with AMPA/kainate, NMDA, and mGluR antagonists showed that the NMDA receptor, but not AMPA/kainate or metabotropic GLUT receptor, antagonism decreased the number of tyrosine hydroxylase-positive DA mesencephalon neurons (Fortin, et al., 2012). These studies suggest that GLUT, and the

NMDA receptor in particular, may be modulating DA activity within areas of the mesocorticolimbic pathway.

Mechanisms of Stress-induced Changes in the Brain

Stress activates the hypothalamus-pituitary-adrenal (HPA) axis by stimulating the paraventricular nucleus of the hypothalamus to release corticotropin releasing hormone (CRH) and arginine vasopressin onto the anterior pituitary. These hormones act synergistically to stimulate the release of adrenocorticotrophic hormone (ACTH). ACTH then travels from the pituitary gland to the cortex of the adrenal gland via the bloodstream. The adrenal cortex then synthesizes and releases stress hormones known as corticosteroids into the bloodstream. These hormones include cortisol in humans and the equivalent primary corticosteroid hormone corticosterone (CORT) in rodents. Corticosteroids are highly lipophilic and can easily penetrate the cell membrane to bind to intracellular receptors in the hypothalamus, pituitary gland, PFC, or hippocampus to suppress the secretion of CRH and ACTH, thus creating a negative feedback loop. There are two known intracellular corticosteroid receptors as first defined by Reul and de Kloet (1985). The type I, or mineralocorticoid (MR), high-affinity receptor is localized within the hippocampus and the cortex. The type II, or glucocorticoid (GR), receptor is found ubiquitously throughout the brain in neurons and glial cells. CORT has nearly a ten-fold higher affinity for the MR ($K_D = 0.5$ nM) than the GR ($K_D = \sim 2.5-5$ nM; Reul & de Kloet, 1985). The high-affinity MR is fully occupied at low concentrations of CORT; however, when CORT levels rise (i.e. under stressful conditions), the GR becomes occupied (Reul & de Kloet, 1985). In previous work, it was found that dexamethasone, a GR-selective agonist, produced region-specific neuronal loss in the hippocampus (Sousa,

Paula-Barbosa, & Almeida, 1999). Additionally, it has been suggested that activation of the MR may promote neuronal survival and function, whereas activation of the GR may be involved in neuronal degeneration (Gould, Woolley, & McEwen, 1990; Hassan, von Rosenstiel, Patchev, Holsboer, & Almeida, 1996; Sousa, et al., 1999); thus, the damaging effects caused by chronic, high levels of CORT are likely mediated, at least in part, by GR activation.

Although intracellular receptor binding has long been accepted as the primary model of steroid actions, recent evidence suggests that plasma membrane-bound receptors may be responsible for non-classical, non-genomic actions of steroid hormones (as reviewed in Moore & Evans, 1999). In particular, the classic genomic model of steroids could not account for effects that were very rapid, steroid effects that occurred after RNA and/or protein synthesis was blocked, or effects that remained after steroid conjugation with bovine serum albumin (BSA) thereby rendering the steroid impermeable to the plasma membrane (Moore & Evans, 1999). Plasma membrane-bound CORT receptors have been identified in the amphibian (Orchinik, Murray, & Moore, 1991), avian (Breuner & Orchinik, 2009), and mammalian brain (Orchinik, Hastings, Witt, & McEwen, 1997). CORT has been shown to increase spontaneous action current frequency in the rat paraventricular nucleus independent of GR activation (Zaki & Barrett-Jolley, 2002) and BSA-conjugated CORT has been shown to acutely enhance NMDA-induced Ca^{2+} elevations in cultured hippocampal neurons (Takahashi et al., 2002). Additionally, the CORT-induced enhanced locomotor activity was not attenuated by either a protein synthesis inhibitor or intracellular MR/GR antagonists (Sandi, Venero, & Guaza, 1996).

Glucocorticoids have also been shown to exert effects within the mesocorticolimbic pathway. Many studies have investigated the effects of chronic stress on DA release within areas of the mesocorticolimbic DA reward pathway using *in vivo* microdialysis. Research has shown stress-induced reductions in both the dopamine transporter (DAT) and in vesicular monoamine transporter 2 (VMAT2) function in rodents exposed to daily swim stress for 3 weeks (Zucker, Weizman, & Rehavi, 2005). Piazza and colleagues (1993) showed that animals will self-administer various doses of CORT (25-50 µg/infusion), including doses that result in CORT plasma concentrations comparable to those observed during stressful conditions (i.e. those during restraint stress; 35-45 µg/100 ml). Intermittent tail-shock stress has also been shown to increase the amount of extracellular DA found in the NAcc, striatum, and medial frontal cortex (125-195% basal conditions; Abercrombie, Keefe, DiFrischia, & Zigmond, 1989). Further, Tidey and Miczek (1996a) showed that animals exposed to stressors (i.e. previous social defeat stress) had increased mesocorticolimbic DA release (~160% baseline in NAcc and PFC) during social threat. Similarly, extracellular levels of DA in the NAcc shell, but not the core, were increased in rats immediately following a mild footshock stressor (Kalivas & Duffy, 1995). Removal of endogenous glucocorticoids, via adrenalectomy, resulted in decreased extracellular DA concentrations in the NAcc shell (but not the NAcc core), both at baseline conditions as well as following either a stress or cocaine challenge (Barrot et al., 2000). Although the exact mechanism behind CORT-induced DA release is not well understood, it may, at least in part, be mediated by corticosteroid receptors found on VTA neurons within the mesocorticolimbic DA reward pathway (Harfstrand et al., 1986; Piazza, et al., 1993). Further, administration of a MR

antagonist had no effect on basal DA in the shell of the NAcc, while administration of a GR antagonist resulted in decreased basal DA levels in a dose-dependent manner in the same area (Marinelli, Aouizerate, Barrot, Le Moal, & Piazza, 1998). Taken together, these results indicate that glucocorticoids can produce increases in extracellular DA content within the shell of the NAcc via the GR.

Although the DA pathway appears to be involved in mediating the effects of chronic stress, stress is also thought to exert effects on the glutamatergic NMDA receptor. The ionotropic glutamatergic NMDA receptor is highly permeable to Ca^{2+} and thought to be involved in several models of Ca^{2+} -dependent excitotoxicity, including ischemia/hypoxia, traumatic brain injury, and the effects of ethanol withdrawal. Both metabotropic and ionotropic GLUT receptors, and in particular the NMDA receptor and the NMDA receptor 2B subunit (NR2B), have been shown to mediate glucocorticoid-induced apoptosis in hippocampal cultures (Lu, Goula, Sousa, & Almeida, 2003). Lu et al. showed that the GR agonist dexamethasone (1 μM) produced significant apoptotic toxicity; this effect was significantly attenuated by a 15-min pretreatment with either MK801 (general NMDA receptor antagonist; 10 μM) or ifenprodil (an antagonist at the NR2B subunit; 10 μM). Chronic CORT has been shown to increase levels of mRNA for the NMDA receptor subunits 2A and 2B (NR2A and NR2B, respectively), suggesting that the effect of CORT on the hippocampal formation is mediated, at least in part, by the NMDA receptor (Weiland, Orchinik, & Tanapat, 1997). Previous research has also shown that while exposure to low levels of CORT is not toxic alone, when given in conjunction with other toxic agents (i.e. NMDA receptor agonist), there is an additive effect of cytotoxicity in the organotypic hippocampal slice culture model (Mulholland,

Self, Harris, Littleton, & Prendergast, 2004a; Mulholland et al., 2006). Further, chronic stress (i.e. unpredictable stress twice per day for 3 weeks) resulted in enhanced vulnerability to CORT-induced excitation in the dentate gyrus of the hippocampus (Karst & Joels, 2003). Interestingly, acute CORT has previously been shown to prolong NMDA receptor mediated Ca^{2+} signaling (Takahashi, et al., 2002). Collectively, these results demonstrate the wide range of effects induced by CORT on both dopaminergic and glutamatergic signaling.

Research has also shown that stress may produce changes in numerous transcription factors, or proteins which bind to DNA and are involved in the regulation of gene transcription from DNA to mRNA. In particular, the leucine zipper family of FOS proteins including c-Fos, Fos B, and Δ Fos B are all thought to be affected by stress (for a review, see Miczek et al., 2011). Several studies have indicated that acute stress (i.e. restraint stress) enhances c-Fos mRNA expression in the hippocampus, cortex, hypothalamus, septum and brainstem (Melia, Ryabinin, Schroeder, Bloom, & Wilson, 1994; Senba & Ueyama, 1997); however, this effect appears to become desensitized following repeated stress (Melia, et al., 1994). In contrast, acute restraint stress increased immunoreactivity of Fos B in limbic areas of the hypothalamic paraventricular nucleus and the ventrolateral septum, while chronic restraint stress (i.e. nine days of repeated restraint stress) also led to elevations of Fos B immunoreactivity in the lateral septum and the basolateral and medial amygdala (Stamp & Herbert, 2001; Stamp & Herbert, 1999). More recent studies from the laboratory of Dr. Eric Nestler have demonstrated that Δ Fos B, the truncated form of Fos B, is also involved in mediating the effects of chronic stress. Perrotti and colleagues (2004) found that c-Fos, Fos B, and Δ Fos B were increased in the

frontal cortex and NAcc following acute restraint stress. Similar to previous findings, western blot analysis showed that the production of c-Fos and full-length Fos B was desensitized following chronic (10 days) restraint stress, while Δ Fos B levels remained elevated at this time (Perrotti, et al., 2004). Further probing via immunohistochemistry studies revealed that Δ Fos B expression was increased in the PFC, NAcc core and shell, basolateral nucleus of the amygdala, and lateral septum following chronic stress (Perrotti, et al., 2004). Recently, the glutamatergic AMPA receptor subunit, GluR2, has been implicated in mediating the effects of chronic stress on Δ Fos B expression, as mice resilient to the deleterious effects of stress have both increased Δ Fos B expression and higher AMPA receptor subunit GluR2 expression in the NAcc (Vialou et al., 2010). Taken together, these studies illustrate the effects of stress on the FOS family of transcription factors within areas of the brain critical to reward.

In addition to the effects of stress on the FOS family of transcription factors, stress can also affect the transcription factor cAMP response element-binding protein (CREB; for a review, see Carlezon, Duman, & Nestler, 2005). Similar to the pattern seen with c-Fos, acute stress appears to activate CREB in a GR-dependent manner, resulting in increased levels of phosphorylated CREB (pCREB; Legradi, Holzer, Kapcala, & Lechan, 1997), while chronic mild footshock stress appears to cause a reduction in pCREB levels in areas such as the PFC and other cortical regions (Trentani, Kuipers, Ter Horst, & Den Boer, 2002). Previous research has also revealed a biphasic pCREB response in limbic areas in response to an acute forced swim stressor, such that an early peak in pCREB immunoreactivity was found around 15 minutes post-stressor, which was then followed by a steep reduction and subsequent secondary rise 6-8 hours post-stressor (Bilang-

Bleuel, Rech, De Carli, Holsboer, & Reul, 2002). Further studies have shown that acute stressors may also produce increased levels of CREB in areas of the mesocorticolimbic reward pathway, including the NAcc shell (Barrot et al., 2002). Additionally, CREB phosphorylation in response to an acute ischemic event was found to be NMDA receptor dependent, as pretreatment with MK-801 attenuated the ischemic-induced increase in pCREB within the hippocampus (Mabuchi et al., 2001). These studies indicate that stress may alter CREB activity, along with other transcription factors and neurotransmitters, in regions of the brain critical to reward and possibly alter subsequent reward-related behaviors including responses to substances of abuse.

Methamphetamine Abuse in the Clinical Population

Methamphetamine (METH) abuse has become an increasingly evident problem in the United States, with nearly 500,000 past month users for non-medical purposes as well as over 125,000 new past year initiates in 2011 (Substance Abuse and Mental Health Services Administration, 2012). Combining the rapid euphoric effects of METH and the ease of at-home production, illicit METH manufacturing and abuse has become a problem particularly in rural areas of the United States, including Kentucky (Kentucky State Police, 2010). METH use has been associated with multiple significant health concerns, including cardiovascular toxicity (i.e. myocardial infarction and atherosclerosis; for a review, see Darke, Kaye, McKetin, & Duflou, 2008), tooth decay (i.e. poor oral hygiene and METH mouth; Hamamoto & Rhodus, 2009), and a higher level of cerebrovascular incidents (i.e. increased risk of ischaemic and haemorrhagic stroke; Darke, et al., 2008). The use of METH has also been associated with many central nervous system (CNS) abnormalities, including decreased hippocampal volume

(Thompson et al., 2004), decreased basal cerebral blood flow (Ances et al., 2011), and increased cortical gray matter loss in the frontal, occipital, temporal, and insular lobes (Nakama et al., 2011; Thompson, et al., 2004). A recent study investigating neurotoxicity in METH abusers following death found a loss of striatal tyrosine hydroxylase (the rate limiting enzyme in dopamine synthesis), DAT, and VMAT2 (Kitamura, 2009). Further, the loss of striatal DAT in chronic METH abusers has been reported to remain upwards of 3 years into abstinence (McCann et al., 1998; Volkow et al., 2001). Although METH use is often associated with dopaminergic deficits (Kitamura, 2009), METH-dependent individuals who were abstinent for less than one month showed decreased frontal gray matter GLUT+glutamine concentrations (Ernst & Chang, 2008), suggesting craving during early abstinence may be due, in part, to depleted glutamatergic systems. In addition, some of the CNS abnormalities have been correlated to decreased cognitive performance (Thompson, et al., 2004; van Holst & Schilt, 2011). Thus, METH abuse represents a serious public health concern with multiple psychological and physiological risks, and the mechanism by which METH produces neurological damage should be further examined.

Mechanisms of METH-induced Changes in the Brain

The primary reinforcing effect of psychostimulants, such as METH, is thought to be dependent on the mesocorticolimbic dopaminergic reward pathway. Stimulants are thought to increase DA levels by preventing reuptake of DA from the synapse (White & Kalivas, 1998). In two recent studies, it was shown that METH, amphetamine, and cocaine elevate the extracellular levels of DA in the NAcc, and that METH also increases extracellular levels of L-3,4-dihydroxyphenylalanine (DOPA), a precursor of DA (Izawa,

Yamanashi, Asakura, Misu, & Goshima, 2006; Shoblock, Maisonneuve, & Glick, 2003). METH is thought to substitute for monoamines at cell surface membrane-bound transporters including DAT, the noradrenaline transporter (NET), and the serotonin transporter (SERT; for a review, see Cruickshank & Dyer, 2009). Although the natural function of these transporters is to remove excess neurotransmitter from the synapse, METH is thought to reverse DAT, NET, and SERT, resulting in increased levels of DA and other monoamines in the synapse. METH works in a similar manner at the vesicular monoamine transporter 2 (VMAT2), which is responsible for packaging neurotransmitters into vesicles. At this transporter, METH acts to produce increased levels of cytosolic monoamines and also acts at membrane-bound transporters to transfer these cytosolic monoamines into the synapse, resulting in increased DA, noradrenaline, and serotonin in the synaptic cleft available for post-synaptic receptor binding (Cruickshank & Dyer, 2009). Importantly, METH is also a potent inhibitor of monoamine oxidase, which catabolizes DA and other monoamines, resulting in further increases in monoamine levels (Egashira, Yamamoto, & Yamanaka, 1987). These studies demonstrate the importance of the mesocorticolimbic dopaminergic reward pathway in the reinforcing effects of METH and other psychostimulants.

METH has also been shown to exert effects on glutamatergic signaling and receptors. Previous studies have shown that high doses of METH result in increased extracellular GLUT content in the rodent striatum (Abekawa, Ohmori, & Koyama, 1994; Mark, Soghomonian, & Yamamoto, 2004; Nash & Yamamoto, 1992; Stephans & Yamamoto, 1994). Further, investigations using western blot analysis revealed that subacute exposure to METH (4 mg/kg/day for 14 days) resulted in NMDA receptor

subunit 1 (NR1) upregulation in the frontal cortex, while both acute (8 mg/kg) and subacute exposure resulted in upregulation of NR1 in the striatum (Kerdsan, Thanoi, & Nudmamud-Thanoi, 2009). Another recent immunoblotting study showed an upregulation of NR2A expression and no change in NR1 expression when METH was given acutely, and a downregulation of NR1 expression and no change in NR2A expression when METH was given in escalating doses (Simoes et al., 2007). In contrast to the effects of CORT, short term METH given in conjunction with a NMDA receptor agonist had an antagonizing effect in hippocampal cultures, suggesting that acute METH acts as a functional antagonist at the NMDA receptor (Smith et al., 2007). Accordingly, as the authors suggest, chronic NMDA receptor antagonism via METH may lead to compensatory NMDA receptor subunit upregulation and, hence, may increase vulnerability to further insults during METH withdrawal (Smith, et al., 2007). Thus, it appears that METH may exert region-specific effects on glutamatergic signaling, possibly at the NMDA receptor.

Similar to the effects seen with CORT, METH can also produce alterations in a number of transcription factors, including the FOS family. Acute METH (1-40 mg/kg) has been repeatedly shown to induce widespread c-Fos in rodents within areas of the brain such as the NAcc, striatum, hypothalamus, thalamus, amygdala, frontal cortex, and hippocampus (Beauvais, Jayanthi, McCoy, Ladenheim, & Cadet, 2010; Thiriet, Zwiller, & Ali, 2001; Umino, Nishikawa, & Takahashi, 1995). Further, the timecourse associated with increased c-Fos appears to be similar to that seen with acute CORT, in that with both events c-Fos levels revert back to control levels within 60 minutes (Thiriet, et al., 2001). The induction of c-Fos following acute METH has been reported to be dependent

on activation of D1 receptors (Beauvais, et al., 2010) as well as NMDA receptors (Ohno, Yoshida, & Watanabe, 1994). A recent study demonstrated that, unlike the pattern seen with chronic CORT, chronic METH does not produce desensitization of c-Fos (Cornish, Hunt, Robins, & McGregor, 2012). However, repeated METH (10 mg/kg every 2 hours x 4) did increase FosB expression threefold, although the response was delayed in comparison to that seen with c-Fos (Beauvais, et al., 2010). Similar to the changes in c-Fos, chronic METH self-administration (21 days) resulted in increased FosB expression in the VTA, amygdala, hypothalamus, and dorsal raphe (Cornish, et al., 2012); these changes were also postulated to be a result of increased Δ Fos B due to the long-lasting nature. Although few studies have examined Δ Fos B and METH, expression of Δ Fos B is increased in the NAcc of rats following a three-day withdrawal from METH administration, suggesting a long-lasting change following METH administration (McDaid, Graham, & Napier, 2006). In an elegant review of Δ Fos B and drug addiction, Nestler et al. (2001) propose a timecourse for the activation of FOS family of transcription factors in response to drugs of abuse, with acute drug administration producing rapid increases in c-Fos (peak at 2 hours), followed by the induction of Fos B (~6 hours) and Δ Fos B (up to 12 hours); in contrast, the authors propose an accumulation of Δ Fos B over days or weeks following chronic drug administration. These studies demonstrate the effects of psychostimulants, such as METH, on the expression of the FOS family of transcription factors in regions of the mesocorticolimbic reward pathway.

In addition to affecting the FOS family of transcription factors, METH, like stress, can also affect the transcription factor CREB and pCREB. An acute METH challenge *in vitro* (1-100 μ M) dose-dependently increased CREB binding (Asanuma,

Hayashi, Ordonez, Ogawa, & Cadet, 2000). Similar to the pattern seen with chronic CORT and CREB, chronic METH *in vivo* resulted in decreased expression of CREB in the rodent striatum (McCoy et al., 2011). Expression of pCREB was increased in the frontal cortex of rats following a three-day withdrawal from chronic METH administration, but pCREB expression was decreased in the NAcc at 14 days withdrawal (McDaid, et al., 2006). Although few studies have examined the interaction of pCREB and METH, one study showed that an acute cocaine challenge (10 mg/kg) increased pCREB levels in the NAcc (Walters, Kuo, & Blendy, 2003). Further, increasing CREB expression in the shell of the NAcc produced an upward and leftward shift in the cocaine self-administration fixed ratio dose-response curve as well as increased progressive ratio for cocaine self-administration (Larson et al., 2011). Taken together, these studies illustrate the possible biphasic effects of psychostimulants, like METH, on CREB expression as well as how increased CREB in brain areas of reward may affect subsequent drug abuse.

METH Toxicity

Chronic, high-dose METH abuse is often associated with dopaminergic and non-dopaminergic deficits in multiple brain regions in the clinical population (see Methamphetamine Abuse in the Clinical Population section above), and many preclinical studies have also noted long-lasting METH-induced terminal damage with a large single dose or repeated high doses (see Krasnova & Cadet, 2009 for a review). It has been postulated that METH toxicity (i.e. nerve terminal damage and apoptosis) is a consequence of numerous events, including oxidative stress via increased extracellular DA, excitotoxicity via increased extracellular GLUT, hyperthermia, mitochondrial

dysfunction, neuroinflammatory responses, and endoplasmic reticulum stress (reviewed in Krasnova & Cadet, 2009; Yamamoto, Moszczynska, & Gudelsky, 2010). The METH-induced increase in extracellular DA may result in the formation of reactive oxygen species (ROS) and oxidative stress via auto-oxidation of DA, and administration of antioxidants has been shown to be protective against METH-induced dopaminergic terminal depletion (for reviews, see Kita, Wagner, & Nakashima, 2003; Krasnova & Cadet, 2009). Multiple studies have shown that antagonists of the NMDA receptor such as MK-801 can attenuate the initial increase in extracellular DA and subsequent depletion of dopaminergic terminals following binge METH exposure (Boireau, Bordier, Dubedat, & Doble, 1995; Marshall, O'Dell, & Weihmuller, 1993; O'Callaghan & Miller, 1994; Ohmori, Koyama, Muraki, & Yamashita, 1993; Sonsalla, Riordan, & Heikkila, 1991), suggesting that both DA and GLUT may play a role in the nerve terminal degeneration produced by binge METH.

In addition to a loss of monoaminergic terminals, METH is also thought to cause excitotoxicity and neuronal apoptosis (for a review, see Cadet, Jayanthi, & Deng, 2003). Although the exact mechanism of METH-induced apoptosis is unknown, it has been postulated that chronic METH leads to increased GLUT release and increased Ca^{2+} signaling via the glutamatergic NMDA receptor, which then activates apoptotic cascades (i.e. calpains and caspases) leading to cell death (for a review, see Cadet & Krasnova, 2009; Davidson, Gow, Lee, & Ellinwood, 2001). Consistent with this theory, a recent review found that acute METH activates calpain and caspase cell death cascades in a manner similar to traumatic and ischemic brain injury in rodents (Gold et al., 2009). Further, a single dose of METH (10-40 mg/kg) produced significant increases in calpain

and caspase activation, as indicated by enhanced spectrin and tau protein fragments, respectively, in the rat cortex and hippocampus, with samples collected 24 hours after the highest dose of METH producing fragments equal to that seen in traumatic brain injury (Warren et al., 2005). Additionally, blockade of the NMDA receptor prevented calpain and caspase-3 activation in a hippocampal model of traumatic brain injury (DeRidder et al., 2006). Taken together, these studies implicate the NMDA receptor in both nerve terminal damage and apoptosis resulting from high-dose METH, suggesting it may be a potential target for METH-induced brain damage.

Stress and METH

It has long been suggested that stress and increased production of glucocorticoids may augment the effects of drugs of abuse by selectively enhancing DA release within the NAcc shell (for a review, see Marinelli & Piazza, 2002). During times of chronic stress, an increase in glucocorticoids and DA (via repeated activation of the mesocorticolimbic DA reward pathway) may be a coping mechanism for the aversive effects associated with repeated stress and, thus, could lead to a sensitized state, leaving the organism more vulnerable to the drug-induced rewarding effects and subsequent addiction (Marinelli & Piazza, 2002). In support of this theory, previous stress has been shown to enhance drug-induced behavioral responses such as locomotor activity and stereotypy, the acquisition and maintenance of drug self-administration, drug-induced conditioned place preference, and potentiate stimulant-induced striatal DA levels (see Briand & Blendy, 2010 for a review). For instance, rodents exposed to repeated social defeat stress acquired cocaine self-administration in nearly half the time compared to their non-defeated counterparts, likely due to enhanced extracellular DA levels seen in

the NAcc (Tidey & Miczek, 1996b, 1997). These studies suggest that prior exposure to chronic stressors may lead to an enhanced DA release following administration of many drugs of abuse.

In conjunction with producing an enhancement of METH-induced DA, chronic stress or exposure to CORT may increase the METH-induced toxicity associated with DAT and other receptors. Animals exposed to 10 days of unpredictable stress and subsequently exposed to a neurotoxic regimen of METH showed enhanced extracellular striatal GLUT, increased long-term depletions in striatal DA and serotonin tissue concentrations, and decreased immunoreactivity of DAT and the serotonin transporter in the striatum (Matuszewich & Yamamoto, 2004b; Tata & Yamamoto, 2008); further, these effects appear to be mediated by the classical genomic effects of CORT as administration of the CORT synthesis inhibitor metyrapone prior to each stressor attenuated the combined effects of stress and METH. CORT has previously been shown to increase non-stimulated (i.e. basal) levels of ROS in hippocampal explants (McIntosh & Sapolsky, 1996), suggesting that CORT could enhance METH-induced production of ROS. Importantly, recent research has indicated that exposure to chronic CORT significantly enhanced METH-induced dopaminergic nerve terminal damage and neuroinflammation (Kelly, Miller, Bowyer, & O'Callaghan, 2012). Collectively, these studies suggest that previous exposure to chronic CORT may enhance the amount of METH-stimulated DA and subsequently lead to increased oxidative stress and nerve terminal damage.

Stress, Methamphetamine and the NMDA Receptor

Given the evidence for interactions between stress, METH, and GLUT, it is surprising that few studies have been conducted on the subject. One study showed that 10 days of chronic, unpredictable stress resulted in increased METH-induced DA release and long-term depletions of DA neurons in the striatum, suggesting that stress may leave the brain more vulnerable to future excitotoxic insults such as psychostimulant exposure (Matuszewich & Yamamoto, 2004a). Recently, it was shown that chronic unpredictable stress resulted in enhanced GLUT release in the hippocampus (Raudensky & Yamamoto, 2007a) and in the striatum (Tata & Yamamoto, 2008) following a METH challenge. Further, the NMDA receptor is essential for neurochemical sensitization following repeated METH (Nakagawa et al., 2011), though no study has examined if this translates to repeated stress-induced neurochemical sensitization. Thus, the mechanism behind stress-induced vulnerability to enhanced toxicity produced by psychostimulant use and relapse may lay in the glutamatergic NMDA receptor.

Experimental Rationale

With no current pharmacological treatment for METH dependence (Chen, Wu, Zhang, & Hashimoto, 2010), it is of utmost importance to identify the mechanism by which METH causes toxicity and if that toxicity is exacerbated with continued stress. The mesocorticolimbic reward pathway appears to be critical in mediating the effects of both stress and drugs of abuse such as METH. The current set of studies tested the involvement of the NMDA receptor in CORT-mediated changes in neuronal viability, NMDA receptor subunit levels, and DA/GLUT levels in response to an excitotoxic

challenge in an *in vitro* model of the DA reward pathway. Experiment 1 tested the hypothesis that chronic CORT sensitizes the mesolimbic DA reward pathway to NMDA-induced insults in a GR- and NMDA receptor-dependent manner, as evidenced by increased levels of propidium iodide (PI) uptake (as a measure of cell death/damage) and decreased levels of neuronal nuclear protein (NeuN; to detect healthy mature neurons) immunoreactivity. Given that METH is a known excitotoxin, experiment 2A tested the hypothesis that chronic CORT exposure potentiates METH-induced neurotoxicity in a NMDA receptor-dependent manner, as evidenced by CORT+METH producing increased PI uptake and decreased levels of NeuN immunoreactivity. Experiment 2B tested the hypothesis that chronic CORT and acute METH act synergistically to produce increased expression of the NR1 and NR2B NMDA receptor subunits. Experiment 2C examined the hypothesis that antagonism of the NMDA receptor would prevent the CORT-induced potentiation of METH-evoked DA and GLUT overflow. Levels of DA and GLUT were assessed using high-performance liquid chromatography (HPLC).

Chapter 2

Methods – Experiment 1

1.1 Organotypic Double Co-culture Preparation

In vitro work is well suited to examine CORT-induced differences, as *in vivo* studies with rodents are confounded by diurnal fluctuations in CORT concentrations and *in vitro* models allow for precise control over CORT concentrations. In addition, though the effects of *in vitro* aging on receptor density have not yet been examined in co-cultures of the NAcc and VTA, Martens and Wree (2001) noted that NMDA receptor distribution was comparable in hippocampal slices aged *in vitro* and those taken from aged-matched rats and previous studies have found that a number of synaptic components remain at a steady level following a brief initial depression (Bahr et al., 1995). Additionally, numerous studies have investigated the use of long-term organotypic co-cultures of the VTA/NAcc and reported intact morphology, re-innervation of dopaminergic tracts into the NAcc, and survival for up to 3 months (Jaumotte & Zigmond, 2005; Lyng, Snyder-Keller, & Seegal, 2007; Ostergaard, Schou, & Zimmer, 1990). Thus, organotypic cell culture studies allow for long-term and detailed manipulations investigating mechanisms of brain activity while minimizing environmental influences and may provide a better understanding of CORT-NMDA interactions *in vivo*. Eight day old male and female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were humanely sacrificed and the brains aseptically removed. Following removal, brains were transferred to ice-cold dissecting media (4°C), composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 µM

streptomycin/penicillin (Invitrogen). Following aseptical removal, brains were sliced mid-sagittally and sectioned coronally at 400 μm thickness using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). After sectioning, slices were placed into culture medium, containing dissecting medium along with distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% (v/v) Hanks' Balanced Salt Solution (HBSS; Invitrogen) and 25% heat-inactivated horse serum (HIHS; Sigma) and 0.05% streptomycin/penicillin (Invitrogen). Using a dissecting microscope, intact slices containing the NAcc and VTA were identified, yielding approximately 4-6 slices of each region per animal. Two pair of co-cultures (each pair containing one NAcc and one VTA in slight contact) was plated onto Millicell-CM 0.4 μm biopore membrane inserts (Fisher) with 1 mL of pre-incubated culture media added to the bottom of each well of a six well plate. The striatal slices also included a strip of pre-motor cortex that was not analyzed. Using the dissecting microscope, slices were oriented such that the NAcc and VTA were in slight contact with each other. Plates were then incubated at 37°C with a gas composition of 5%CO₂/95% air for 21 days to allow the co-cultures to grow together and attach to inserts. During this time, the culture media was refreshed every 3 days. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

1.2 Characterization of Double Co-culture Model

1.2a Autoradiography for Dopamine Transporter

Several measures were taken in order to better characterize the NAcc/VTA co-cultures. Autoradiography was conducted for qualification of dopamine transporter binding sites in control-treated cultures. Following 21 days of continuous culture media exposure, co-cultures were flash-frozen in isopentane (Sigma) on dry ice and stored at -80°C until [¹²⁵I]RTI 55-940 binding studies were completed. For [¹²⁵I]RTI 55-940 binding, the Teflon membranes on which the co-cultures were maintained were excised and secured onto glass slides. The glass slides containing the co-cultures were then preincubated for 10 minutes in 50 mM Tris-HCl (Sigma) containing 3 mM MgCl₂ (Sigma), 0.2 mM ethylene glycol tetraacetic acid (Sigma), and 100 NaCl (Sigma) at room temperature. Following preincubation, tissue was then incubated with [¹²⁵I]RTI 55-940 (0.01 nM) at room temperature for 120 minutes in the above Tris-HCl solution also containing 30 nM fluoxetine (Sigma). After the 120 minute radioligand incubation period, cultures were washed with Tris-HCl wash buffer for 1 minute, followed by two 20-minute wash periods, at 4°C. Following washing, cultures were dipped in water at 4°C and allowed to dry overnight. Slides containing the cultures were then placed into a light-proof cassette and exposed to Kodak film (Fisher). Films were developed following approximately 3 days of exposure.

1.2b Immunohistochemistry of Tyrosine Hydroxylase and Myelin Basic Protein

In addition to autoradiography, immunohistochemistry for tyrosine hydroxylase (the rate limiting enzyme in the synthesis of dopamine) and myelin basic protein (to stain myelinated axon fibers) was also conducted on control-treated co-cultures. Following 21 days of exposure to control media, co-cultures were fixed by transferring the insert containing the co-culture to a plate containing 1 mL of 10% formalin solution on the

bottom of each well. One mL of formalin was also placed on top of the insert and the plates were allowed to sit for 30 minutes. The slices were then washed carefully (1 mL on bottom of the well and 1 mL on top of the well) with 1 X phosphate buffered saline (PBS) twice and stored with 1 mL of 1 X PBS on the bottom of the well overnight at 4°C. Following overnight storage, the inserts were transferred to a plate containing 1 mL of membrane permeabilization buffer (200 mL 1 X PBS (Invitrogen), 200 µL Triton X-100 (Sigma), 0.010 mg Bovine Serum (Sigma)) in each well and 1 mL of permeabilization buffer was also placed on top of each insert containing the slices. The slices were allowed to sit in buffer for 45 minutes to allow the buffer to penetrate the slice and were then washed twice with 1 X PBS as described earlier. Inserts were then transferred to a plate containing 1 mL of 1 X PBS on the bottom of each well and were treated with 1 mL of permeabilization buffer containing mouse anti-tyrosine hydroxylase (1:200; Sigma) and rabbit anti-myelin basic protein (MBP; 1:100; Sigma) on top of each well. Plates were then stored at 4°C for 24 hours. Following 24 hours, the slices were washed gently with 1 X PBS twice and were again transferred to a plate containing 1 mL of 1 X PBS on bottom. At this point, slices were treated with 1 mL of permeabilization buffer containing the goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC; 1:100; Sigma) for tyrosine hydroxylase-labeled cultures and the goat anti-rabbit secondary antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC; 1:200; Sigma) for myelin basic protein-labeled cultures on top of the insert and were stored at 4°C for 24 hours. After 24 hours, slices were washed twice with 1 X PBS as described previously and placed into a plate containing 1 mL of 1 X PBS on the bottom of each well. The slices were imaged immediately with PBS under each insert.

Co-cultures were visualized with SPOT advanced version 4.0.2 software for Windows (W. Nuhsbahr Inc.; McHenry, IL, USA) using a 2.5x objective with a Leica DMIRB microscope (W. Nuhsbahr Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) using blue-green light and connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsbahr). The secondary antibody with FITC was elicited using a band-pass filter at 495 nm (520 nm emission) while the secondary antibody conjugated to TRITC was elicited using a band-pass filter that excites wavelengths between 515-560 nm (620 nm emission).

1.3 CORT pre-exposure

Male and female co-cultures (n=2/well) were continuously exposed to physiologically relevant corticosterone (CORT; 1 μ M; Sigma) for 5 days beginning at 21 days *in vitro* (DIV), followed by a 24 hour co-exposure to NMDA. At 21 DIV, co-cultures were randomly transferred to plates containing either 1 mL of culture media with 0.2% dimethyl sulfoxide (DMSO: vehicle control; Fisher) or 1 mL of culture media containing 1 μ M of CORT. For studies investigating the potential role of the GR, a subset of these cultures was also exposed to the GR antagonist mifepristone (10 μ M; Sigma) for 5 days beginning at 21 DIV. For all experiments, CORT and mifepristone stock solutions were dissolved in 100% DMSO and diluted with culture media to the desired concentration so as to yield a final concentration of 0.2% DMSO. Concentrations of CORT and mifepristone were chosen based on physiological relevance to exposure to a variety of stressors as well as previous organotypic experiments (Bielajew, Konkle, & Merali, 2002; Livezey, Miller, & Vogel, 1985; Mulholland, Self, Harris, Littleton, & Prendergast, 2004b; Sharrett-Field, Butler, Berry, Reynolds, & Prendergast, 2013). All

six-well plates were then returned to an incubator maintained at 37°C with a gas composition of 5%CO₂/95% air. This exact treatment regimen was repeated at 24 DIV.

1.4 NMDA co-exposure

At 26 DIV co-cultures were transferred to new plates containing either 1 mL of culture media with CORT or vehicle (as previously described) or 1 mL of culture media with CORT/vehicle along with NMDA (200 µM; Sigma). This concentration of NMDA was chosen based on previous reports suggesting that higher concentrations (>100 µM) are necessary for NMDA-induced cytotoxicity in co-cultures containing the VTA-NAcc pair (Maeda, Ibi, Shimazu, & Akaike, 1998). To test the involvement of the NMDA receptor, a subset of cultures was also co-exposed to the NMDA receptor antagonist 2-amino-7-phosphonovaleric acid (APV; 50 µM; Sigma), or NMDA+APV (as above). For studies investigating the potential role of the GR, a second subset of co-cultures was also co-exposed to NMDA and the GR antagonist mifepristone (as described above). In addition, all culture media contained propidium iodide (PI; 3.74 µM; Molecular Probes, Eugene, OR) in order to assess cytotoxicity.

1.5a Cytotoxicity Assessment via Propidium Iodide

Cytotoxicity (PI staining of neurons and glia with damaged membranes) was evaluated in all regions of the co-cultures using fluorescent microscopy. Previous literature has demonstrated that PI reliably correlates with other measures of cell death, including fluoro-jade staining for histology and the release of lactate dehydrogenase within the culture media (for a review, see Zimmer, Kristensen, Jakobsen, & Noraberg, 2000). Co-cultures were visualized with SPOT advanced version 4.0.2 software for

Windows (W. Nuhsbahr Inc.; McHenry, IL, USA) using a 2.5x objective with a Leica DMIRB microscope (W. Nuhsbahr Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) using blue-green light and connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsbahr). PI has an emission wavelength of 620 nm in the visual range and a peak excitation wavelength of 536 nm and was excited using a band-pass filter that excites a range of wavelengths (510-560 nm). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was used to measure the intensity of the PI fluorescence. A background measurement was also taken from the visual field surrounding each co-culture and was subsequently subtracted from the region measurements of each co-culture. The intensity was measured in each of the two regions of interest: the NAcc and the VTA. Within each region, measurements of PI uptake were converted to percent control for comparison across repetitions.

1.5b Cytotoxicity Assessment via Immunohistochemical Analysis of the Neuronal Nuclear Protein (NeuN)

Following imaging for PI uptake, co-cultures were subsequently fixed to assess immunoreactivity of the neuronal nuclear protein (NeuN) according to methods detailed above in section 1.2b except that slices were incubated overnight with 1 mL of permeabilization buffer containing mouse anti-NeuN (1:200; Millipore, Billerica, MA) on top of each well. Recently, NeuN has also been classified as Fox-3, a member of the Fox-1 family of splicing regulators for pre-mRNA (Kim, Adelstein, & Kawamoto, 2009). Following two washes with 1 X PBS as described above, slices were treated with 1 mL of permeabilization buffer containing the goat anti-mouse secondary antibody conjugated to

fluorescein isothiocyanate (FITC; 1:100; Sigma) on top of the insert and were stored at 4°C for 24 hours. After 24 hours, slices were washed twice with 1 X PBS as described previously and placed into a plate containing 1 mL of 1 X PBS on the bottom of each well. The slices were imaged immediately with PBS under each insert. The slices were visualized as described previously above; the secondary antibody with FITC was excited using a band-pass filter at 495 nm (520 nm emission). Densitometry using Image J software (National Institutes of Health) was used to measure the intensity of the FITC fluorescence. A background measurement of fluorescence was taken from the visual field surrounding each slice and was subsequently subtracted from the region measurement of each slice before analysis. The intensity was measured in each of the two regions of interest: the ventral tegmental area and the nucleus accumbens. To control for the variability between each replication, measurements of FITC immunoreactivity were converted to percent control for each region before statistical analysis.

1.6 Statistical Analysis

Each well contained two co-cultures and each co-culture was analyzed individually. All treatment effects were examined using a two-way analysis of variance (ANOVA; treatment X sex). Each experiment was conducted a minimum of 3 times using different rat litters, with each experimental condition containing 4 co-cultures. A total of 8 different rat litters were used for these experiments, totaling a range of 4-16 slices per sex per treatment group. Data from each replication was converted into percent control values and was subsequently combined if litter effects were absent. When appropriate, post-hoc tests were conducted using Fisher's LSD to examine further effects. Statistical significance was set at $p < 0.05$.

Methods – Experiment 2

2.1 Organotypic Triple Co-culture Preparation

In vitro work is acutely suited to examine CORT-induced differences, as *in vivo* studies with rodents are confounded by diurnal fluctuations in CORT concentrations. In addition, though the effects of *in vitro* aging on receptor density have not yet been examined in triple co-cultures, Martens and Wree (2001) showed that NMDA receptor distribution was comparable in hippocampal slices aged *in vitro* and those taken from aged-matched rats. Previous studies have found that a number of synaptic components remain at a steady level following a brief initial depression (Bahr, et al., 1995). Additionally, numerous studies have investigated the use of long-term organotypic co-cultures of the VTA/NAcc and reported intact morphology, re-innervation of dopaminergic tracts into the NAcc, and survival for up to 3 months (Jaumotte & Zigmond, 2005; Lyng, et al., 2007; Ostergaard, et al., 1990). Thus, organotypic cell culture studies allow for long-term and detailed manipulations investigating mechanisms of brain activity while minimizing environmental influences and may provide a better understanding of CORT-METH interactions *in vivo*. Eight day old male and female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were humanely sacrificed and the brains aseptically removed. Following removal, brains were transferred to ice-cold dissecting media (4°C), composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 µM streptomycin/penicillin (Invitrogen). Following aseptic removal, the brains were sliced mid-sagittally and subsequently sectioned coronally at 400 µm thickness using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK).

After sectioning, slices were placed into culture medium, containing dissecting medium along with distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% (v/v) Hanks' Balanced Salt Solution (HBSS; Invitrogen) and 25% heat-inactivated horse serum (HIHS; Sigma) and 0.05% streptomycin/penicillin (Invitrogen). Using a dissecting microscope, intact slices containing the NAcc, VTA, or mPFC were identified. Co-cultures containing one slice from each NAcc, VTA, and mPFC were plated onto Millicell-CM 0.4 μ m biopore membrane inserts with 1 mL of pre-incubated culture media added to the bottom of each well of a six well plate. Using the dissecting microscope, slices were oriented such that the NAcc, VTA, and mPFC are in slight contact with each other (Figure 1A). Plates were then incubated at 37°C with a gas composition of 5%CO₂/95% air for 21 days to allow the triple slice co-cultures to grow and attach to inserts. During this time, the culture media was changed every 3 days. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

2.2 Characterization of triple co-culture model

2.2a Immunoreactivity of myelin basic protein

In order to better characterize the triple slice co-cultures, immunohistochemistry for myelin basic protein (to stain myelinated axon fibers) was conducted on control-treated co-cultures. Following 21 days of exposure to control media, co-cultures were fixed by transferring the insert containing the co-culture to a plate containing 1 mL of 10% formalin solution on the bottom of each well. One mL of formalin was also placed

on top of the insert and the plates were allowed to sit for 30 minutes. The slices were then washed carefully (1 mL on bottom of the well and 1 mL on top of the well) with 1 X phosphate buffered saline (PBS) twice and stored with 1 mL of 1 X PBS on the bottom of the well overnight at 4°C. Following overnight storage, the inserts were transferred to a plate containing 1 mL of membrane permeabilization buffer (200 mL 1 X PBS (Invitrogen), 200 µL Triton X-100 (Sigma), 0.010 mg Bovine Serum (Sigma)) in each well and 1 mL of permeabilization buffer was also placed on top of each insert containing the slices. The slices were allowed to sit in buffer for 45 minutes to allow the buffer to penetrate the slice and were then washed twice with 1 X PBS as described earlier. Inserts were then transferred to a plate containing 1 mL of 1 X PBS on the bottom of each well and were treated with 1 mL of permeabilization buffer containing rabbit anti-myelin basic protein (1:100; Sigma) on top of each well. Plates were then stored at 4°C for 24 hours. Following 24 hours, the slices were washed gently with 1 X PBS twice and were again transferred to a plate containing 1 mL of 1 X PBS on bottom. At this point, slices were treated with 1 mL of permeabilization buffer containing the goat anti-rabbit secondary antibody conjugated to fluorescein isothiocyanate (FITC; 1:100; Sigma) for myelin basic protein-labeled cultures on top of the insert and were stored at 4°C for 24 hours. After 24 hours, slices were washed twice with 1 X PBS as described previously and placed into a plate containing 1 mL of 1 X PBS on the bottom of each well. The slices were imaged immediately with PBS under each insert. Co-cultures were visualized with SPOT advanced version 4.0.2 software for Windows (W. Nuhsbalm Inc.; McHenry, IL, USA) using a 2.5x objective with a Leica DMIRB microscope (W. Nuhsbalm Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) using blue-green light and

connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsburg). The secondary antibody with FITC was elicited using a band-pass filter at 495 nm (520 nm emission).

2.2b NMDA-induced Cytotoxicity via Propidium Iodide Uptake

Following 21 days of exposure to control culture media, co-cultures were treated with culture media containing propidium iodide (PI) and the GLUT agonist NMDA (200 μ M). PI staining of neurons and glia with damaged membranes was evaluated 24 hours later using fluorescent microscopy. It has been shown previously that PI reliably correlates with other measures of cell death, including fluoro-jade staining for histology and the release of lactate dehydrogenase within the culture media (for a review, see Zimmer, et al., 2000). Co-cultures were visualized with SPOT advanced version 4.0.2 software for Windows (W. Nuhsbalm Inc.; McHenry, IL, USA) using a 2.5x objective with a Leica DMIRB microscope (W. Nuhsbalm Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) using blue-green light and connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsburg). PI has an emission wavelength of 620 nm in the visual range and a peak excitation wavelength of 536 nm and was excited using a band-pass filter that excites a range of wavelengths (510-560 nm).

2.3 CORT Pre-exposure in Co-cultures

Male and female triple slice co-cultures (n=2/well) were continuously pre-exposed to CORT for 5 days beginning at 21 days in vitro (DIV). At that time, co-cultures were randomly transferred to plates containing either culture media with 0.1%

DMSO (vehicle control) or culture media containing CORT (1 μ M) dissolved in 0.1% DMSO. Plates were then returned to an incubator maintained at 37°C with a gas composition of 5%CO₂/95% air. This treatment was repeated at 24 DIV for a total of 5 day pre-exposure to CORT.

2.4 METH Exposure in Co-cultures

Male and female triple slice co-cultures (n=2/well) were continuously pre-exposed to CORT for 5 days beginning at 21 DIV. At 26 DIV, co-cultures were co-exposed to CORT/vehicle and METH (0 or 100 μ M; Sigma) in the presence or absence of the NMDA receptor antagonist 2-amino-7-phosphonovaleric acid (APV; 50 μ M). Concentrations of CORT and METH were chosen for their relevance to stress and METH toxicity studies (Aizenman, McCord, Saadi, Hartnett, & He, 2010; Prendergast & Mulholland, 2012; Smith, et al., 2007). Neuronal viability was quantified using PI or NeuN immunoreactivity (experiment 2A; 27-30 DIV) and NMDA receptor subunits were quantified using immunoblotting (experiment 2B) after 24 hour co-exposure to CORT and METH. Additionally, 1 mL of aCSF was placed on top of each insert at the time of the acute METH treatment and collected 45 minutes later for evaluation of DA and GLUT content via HPLC (experiment 2C).

2.5a Cytotoxicity assessment via Propidium Iodide

Cytotoxicity (PI staining of neurons and glia with damaged membranes) was evaluated in all regions of the co-cultures using fluorescent microscopy. It has been shown previously that PI reliably correlates with other measures of cell death, including fluoro-jade staining for histology and the release of lactate dehydrogenase within the

culture media (for a review, see Zimmer, et al., 2000). Co-cultures were visualized with SPOT advanced version 4.0.2 software for Windows (W. Nuhsbalm Inc.; McHenry, IL, USA) using a 2.5x objective with a Leica DMIRB microscope (W. Nuhsbalm Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) using blue-green light and connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsbalm Inc.). PI has an emission wavelength of 620 nm in the visual range and a peak excitation wavelength of 536 nm and was excited using a band-pass filter that excites a range of wavelengths (510-560 nm). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was used to measure the intensity of the PI fluorescence. A background measurement was taken from the visual field surrounding each co-culture and was subsequently subtracted from the regional measurements of each co-culture. The intensity was measured in each of the three regions of interest: the NAcc, the VTA, and the mPFC. Within each region, measurements of PI uptake were converted to percent control for comparison across repetitions. To ensure reliability of PI as a measure of cytotoxicity, NeuN immunoreactivity was also examined as a marker of healthy mature neurons.

2.5b Cytotoxicity Assessment via Immunohistochemical Analysis of the Neuronal Nuclear Protein

Immunohistochemistry was conducted on CORT-naïve, CORT-exposed, METH-naïve, and METH-exposed co-cultures to identify any CORT- or METH-induced changes in NeuN immunoreactivity. Following cytotoxicity analysis, co-cultures were transferred to a plate containing 1 mL of 10% formalin solution on the bottom of each well. One mL of formalin was also placed on top of the insert and the plates were allowed to sit for 30

minutes. The co-cultures were then twice washed carefully (1 mL on bottom of the well and 1 mL on top of the well) with 1 X PBS and stored with 1 mL of 1 X PBS on the bottom of the well overnight at 4°C. Following overnight storage, the inserts were transferred to a plate containing 1 mL of wash buffer (200 mL PBS [Invitrogen], 200 µL Triton X-100 [Sigma], 0.010 mg Bovine Serum [Sigma]) in each well, 1 mL of wash buffer was also placed on top of each insert containing the slices. The co-cultures were allowed to sit in buffer for 45 minutes, allowing the buffer to penetrate the slice, and were then washed twice with 1 X PBS as described earlier. Inserts were transferred to a plate containing 1 mL of 1 X PBS on the bottom of each well and were treated with mouse anti-NeuN antibody (1:200; Millipore) on top of each well. Plates were stored at 4°C for 24 hours. Following 24 hour incubation with the primary antibody, the slices were washed gently with 1 X PBS twice and were again transferred to a plate containing 1 mL of 1 X PBS on bottom. At this point, slices were treated with 1 mL of buffer containing the goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC; 1:100; Sigma) on top of the insert and were stored at 4°C for 24 hours. A range of concentrations was previously examined and the concentrations of NeuN and FITC antibodies chosen were shown to have the least amount of background while still maintaining specificity. After a 24 hour incubation period with the secondary antibody, slices were washed twice with 1 X PBS as described previously and placed into a plate containing 1 mL of PBS on the bottom of each well. The slices were then imaged immediately according to the method below.

The immunofluorescence of each co-culture was visualized as described above (see cytotoxicity assessment) except that the secondary antibodies with FITC was excited

using a band-pass filter at 495 nm (FITC; 520 nm emission). Densitometry using Image J software was again used to measure the intensity of the fluorescence. A background measurement of non-specific fluorescence was taken from the visual field surrounding each co-culture and was subsequently subtracted from the region measurement of each area of interest before analysis. The intensity was measured in each of the three regions of interest: the NAcc, the VTA, and the mPFC. To control for the variability between each replication, measurements of NeuN immunoreactivity were converted to percent control for each region before statistical analysis.

2.6 Western Blot Procedure

While immunohistochemistry has the advantage of investigating regional differences, the thickness of the co-cultures (400 μm) may affect the ability of densitometry to detect subtle differences. Thus, a subset of male and female control, CORT-, and METH-exposed co-cultures were used in immunoblotting analysis. Co-cultures were homogenized in ice-cold lysis buffer containing 62.5 mM trimethylol aminomethane (Fisher), 6 M urea (Sigma), 10% glycerol (Fisher), and 2% sodium dodecyl sulfate (SDS; Sigma) with an adjusted pH of 6.8. Prior to tissue collection, 1 ml of PMSF (phenylmethanesulfonyl fluoride; Sigma) + PBS solution was placed on the bottom of each well in an empty 6 well plate. Stock PMSF (99.8 μM) was first dissolved in 200 proof EtOH and diluted to yield a final concentration of 1:100 (PMSF: 1 X PBS). A protease inhibitor cocktail tablet (Sigma) was added to 0.5 mL lysis buffer and further diluted to a concentration of 1:100 using lysis buffer (v/v). Inserts were then transferred to the plate containing PMSF+PBS and 100 μL of the lysis buffer-protease inhibitor mixture was then placed on top of each well. Tissue was subsequently scraped from the

teflon membrane and placed into 1.5 mL microcentrifuge tubes. The tissue was sonicated for 10-15 seconds at 20% amplitude (Sonics and Materials, Newtown, CT), immediately aliquoted for storage at -80°C until use. A 10 µL aliquot was used to determine the amount of protein within each sample using the Pierce BCA protein assay kit (Fisher).

Based on the results from the protein assay, equal amounts of protein (20 µg) were compared against molecular weight standards (Bio-Rad, Hercules, CA) on 10-lane 4-15% tris-glycine polyacrylamide gels (Bio-Rad). Samples were electrophoresed on ice for 60 minutes at 150 V using the mini-PROTEAN tetra system (Bio-Rad) in 1 X tris/glycine/SDS buffer (Bio-Rad). Following electrophoresis, gels were removed from the apparatus and allowed to soak in transfer buffer (1 X tris/glycine/SDS buffer + 20% methanol) for 5 minutes. Samples were then transferred to 0.45 µM nitrocellulose membrane paper (Bio-Rad) at 100 V for 60 minutes at room temperature in the presence of an ice pack using the mini-PROTEAN tetra system. Following transfer to the nitrocellulose paper, membranes were then washed in 1 X TBS (Bio-Rad) for 15 minutes at room temperature prior to placement in a blocking solution of 5% nonfat dry milk powder in 1 X TBS for 60 minutes at room temperature. Membranes were then rinsed in TTBS (1 X TBS + 0.05% Tween 20 [Fisher]) for 15 minutes at room temperature and subsequently incubated with primary antibodies (mouse anti-NR1, 1:1000, BD Biosciences Pharmingen; rabbit anti-NR2B, 1:1000, Millipore) overnight at 4°C with agitation in a 5% nonfat dry milk powder and TTBS solution. On the second day, membranes were washed for 20 minutes in TTBS and then incubated with the species-appropriate fluorescent secondary antibodies (1:5000, Rockland Immunohistochemicals, Gilbertsville, PA) in light-proof boxes at room temperature for 1 hour with agitation.

Membranes were then washed again for 20 minutes in TTBS and then scanned and quantified using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). Fluorescent intensity was again determined using Image J software and was converted to percent control to compare across treatment groups. This protocol was modified from the laboratories of Dr. James Geddes and Dr. Patrick Mulholland.

2.7 High Performance Liquid Chromatography for Dopamine and Glutamate

Artificial cerebrospinal fluid samples were analyzed for DA and GLUT content using HPLC with electrochemical detection (HPLC-EC, ESA Inc., Chelmsford, MA). The DA HPLC system consisted of a solvent delivery unit, a Coulochem III electrochemical detector equipped with a 5014B analytical cell, and a 5020 guard cell. For DA analysis, the guard cell was set at +350 mV, electrode 1 at -150 mV, and electrode 2 at +220 mV with the gain set to 10 nA. The DA mobile phase consisted of 75 mM NaH₂PO₄, 1.7 nM 1-octanesulfonic acid, 24 μM ethylenediaminetetraacetic acid, 100 μL/L triethylamine and 10.7% acetonitrile (pH was adjusted to 3.0 with phosphoric acid), and was pumped through the system at 0.5 mL/minute. Samples (20 μL) for DA analysis were loaded into a 100 μL loop and injected onto an analytical column (MD-150 3.2mm x 100mm, 3μm Dionex, ThermoScientific, Sunnyvale, CA). The GLUT HPLC system consisted of a solvent delivery unit, a Coulochem III electrochemical detector equipped with a 5011 analytical cell, and a 5020 guard cell. For GLUT analysis, the guard cell was set at +650 mV, electrode 1 at +150 mV, electrode 2 at +550 mV, and the gain was set at 5 μA. Precolumn derivatization of GLUT with o-phthaldialdehyde (OPA; Pickering Lab Inc, CA) and 2-mercaptoethanol (Sigma) was performed using an ESA 542 autosampler. The GLUT mobile phase consisted of 100 mM Na₂HPO₄, 16%

methanol, and 2.5% acetonitrile (pH was adjusted to 6.5 with phosphoric acid), and was pumped through the system at 0.5 mL/minute. GLUT and aspartate were separated using an analytical column (Xterra MS C18 2.5 μ m 3.0 x 50mm column, Waters, Milford, MA). Retention times of external standards were used to determine the corresponding peak height of both DA and GLUT. Chromatograms of samples were then compared against the external standards using an ESA Chromatography Data System (EZChrom Elite, Chelmsford, MA). HPLC analysis was conducted with assistance from the laboratory of Dr. Michael Bardo at the University of Kentucky.

2.8 Statistical Analysis

Each triple slice co-culture was analyzed individually. To test for treatment and sex differences for experiment 1, a two-way analysis of variance (ANOVA; treatment x sex) was investigated for each region of interest: NAcc, VTA, and mPFC. Investigations from experiments 2 and 3 implemented a one-way ANOVA for treatment for western blot and HPLC analysis. Each experiment was conducted a minimum of 3 times using different rat litters. Data from each replication were converted into percent control values and subsequently combined if litter effects were absent. When appropriate, post-hoc tests were conducted using Fisher's LSD to examine further effects. Statistical significance was set at $p < 0.05$.

CHAPTER 3

Results – Experiment 1

Characterization of Double Co-culture Model (Figure 1.1)

Co-cultures of the NAcc and VTA were plated such that the two regions were touching with juncture points at the mid-ventral surface of each culture and allowed to culture for 21 days *in vitro*. Initial studies were completed to characterize the NAcc/VTA co-culture model and possible re-innervation of dopaminergic phenotypes. Figure 1.1A illustrates a light microscope picture indicating the orientation of both the NAcc and VTA as well as the junction of the co-cultures. Densely labeled autoradiography of the dopamine transporter using ^{125}I -RTI-55 binding (Figure 1.1B) was observed in the region bridging the co-cultures. In addition, immunoreactivity of tyrosine hydroxylase (Figure 1.1C; the rate limiting enzyme in the synthesis of catecholamines) and myelin basic protein (Figure 1.1C; to stain myelinated fibers) was also examined with concentrated staining in the region bridging the junction of the co-cultures. Taken together, these results suggest re-innervation within the co-culture model that is likely dopaminergic in nature.

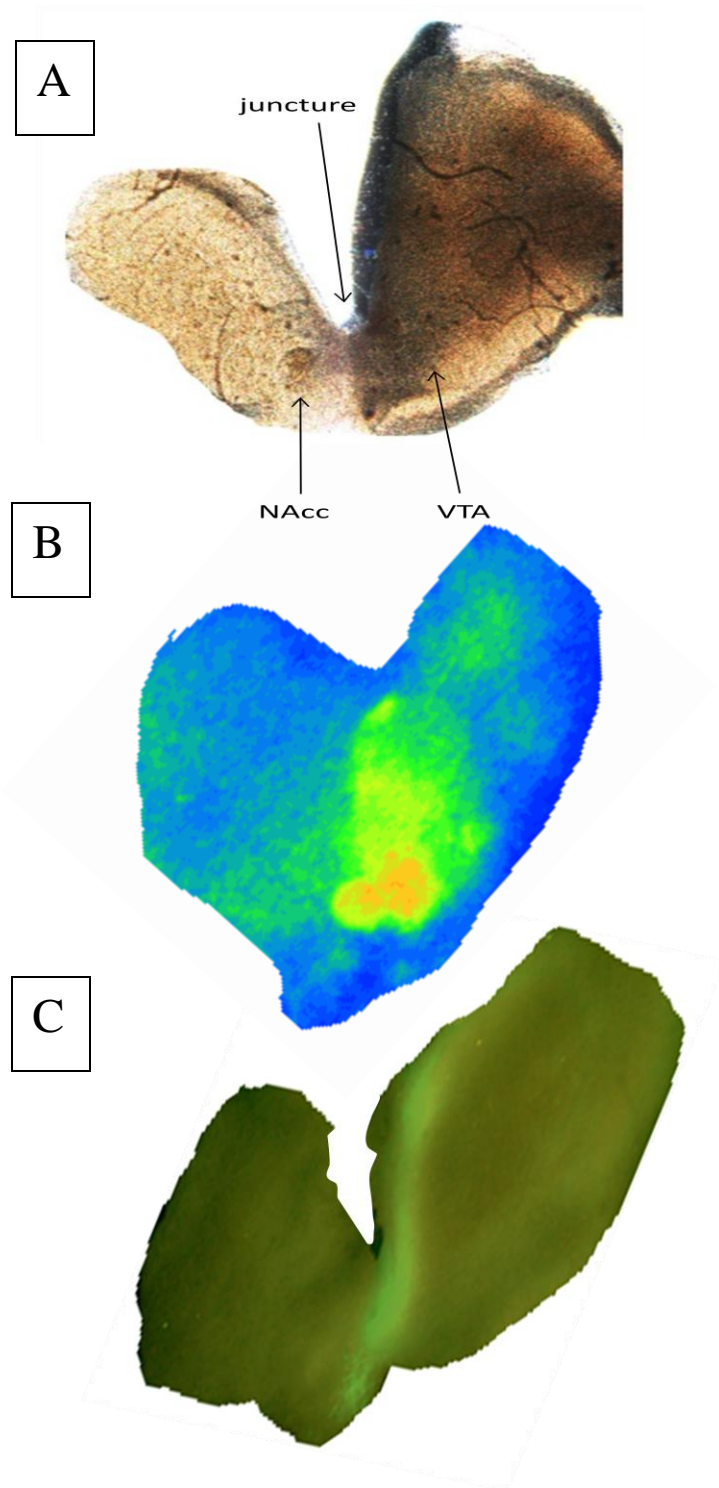


Figure 1.1. Organotypic midbrain-striatal co-cultured ex vivo with ventral tegmental area and nucleus accumbens in contact (2.5x magnification). A. bright-field image of co-cultures; B. Autoradiographic localization of ^{125}I -RTI-55 binding (dopamine transporter) and point of juncture; C. merged image of tyrosine hydroxylase (FITC; green) and myelin basic protein (TRITC; red) immunoreactivity at point of juncture.

Effects of CORT on NMDA-induced Cytotoxicity via Propidium Iodide Uptake (Figures 1.2 and 1.3; Table 1.1)

This set of experiments was designed to assess the neurotoxic effects of 5 days of previous CORT pre-exposure followed by 24 hours of co-exposure to CORT and NMDA, and to investigate the potential roles of the NMDA receptor and the GR in the observed cytotoxicity. There was no difference in toxicity between sexes within each treatment group, and male and female data were combined for further analysis. Thus, a one-way ANOVA for treatment was conducted within each region of interest. In the VTA, a significant main effect of treatment was observed (Figure 1.2; $F(11, 198)=4.277$, $p<0.001$). Regardless of acute treatment (NMDA/APV/mifepristone), vehicle-pretreated co-cultures showed no significant toxicity in the VTA (~95-130% vehicle control; Figure 1.2A). Further, exposure to continuous CORT (for a total of 6 days) in NMDA-naïve tissue produced no significant changes in toxicity compared to vehicle treated co-cultures (~95 % vehicle control; Figure 1.2B). However, significant toxicity was observed in the VTA after 5 days of pre-exposure to CORT followed by 24 hour exposure to CORT+NMDA (~170% vehicle control; Fisher's LSD post-hoc, $p<0.001$). The NMDA-induced toxicity in CORT pre-treated co-cultures was significantly attenuated by co-exposure to APV ($p<0.001$). In regards to the GR, pre-treatment with mifepristone during the 5 day exposure to CORT prior to the 24 hour co-exposure to NMDA significantly reduced CORT+NMDA toxicity ($p<0.01$). Thus, chronic CORT pre-exposure sensitized the VTA to subsequent NMDA receptor-dependent toxicity, and this effect was also dependent on activation of the GR during the CORT pre-exposure. Representative images of these effects are presented in Figure 1.3.

Within the NAcc, a significant main effect of treatment was observed (table 1.1; $F(11, 198)=4.783, p<0.001$). Compared to vehicle alone, 5 days of pre-exposure to either vehicle or CORT in the presence of mifepristone significantly enhanced subsequent NMDA-induced toxicity produced by the 24 hour co-exposure to 200 μM NMDA (~130% vehicle control; $p<0.05$). Exposure to CORT (for a total of 6 days) in NMDA-naïve tissue produced no significant changes in toxicity compared to vehicle treated co-cultures (~96 % vehicle control). However, in contrast to the effects seen within the VTA, there was no significant toxicity observed in co-cultures pre-treated with CORT and later co-exposed to NMDA (~97% vehicle control). Representative images of these effects are presented in Figure 1.3.

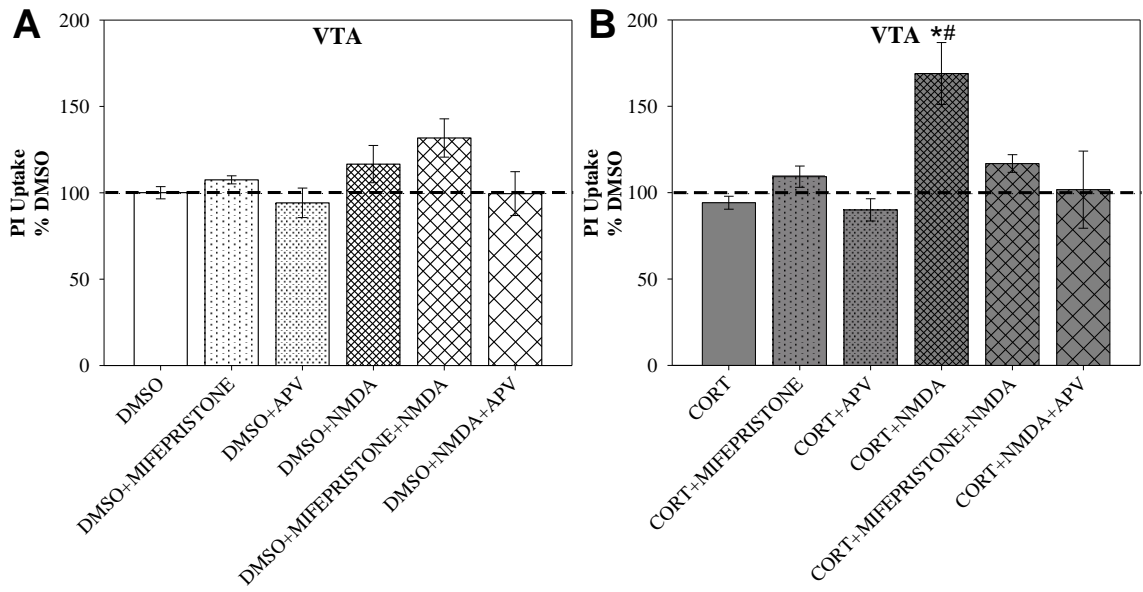


Figure 1.2. Effects of 5 day pre-treatment with either vehicle (DMSO; panel A), 1 μ M CORT (panel B), 10 μ M mifepristone, or CORT + mifepristone prior to 24 hour co-exposure to either vehicle/CORT/mifepristone along with 200 μ M NMDA + 50 μ M APV on PI uptake within the ventral tegmental area (VTA). Pre-treatment with CORT prior to NMDA exposure resulted in significant neurotoxicity, an effect which was not seen in vehicle pre-treated cultures. Co-exposure to the competitive NMDA receptor antagonist APV prevented the NMDA-induced cytotoxicity in CORT pre-treated cultures. Co-exposure to the glucocorticoid receptor antagonist mifepristone during the 5 day CORT pre-treatment and subsequent NMDA exposure also significantly attenuated the ability of CORT to potentiate NMDA-induced toxicity. Dashed line represents DMSO-treated value. Data represented as percentage of DMSO-treated cultures (mean \pm SEM). * $p < 0.05$ vs. DMSO (vehicle) levels; # $p < 0.05$ vs. DMSO+NMDA, CORT+MIFEPRISTONE+NMDA, CORT+NMDA+APV

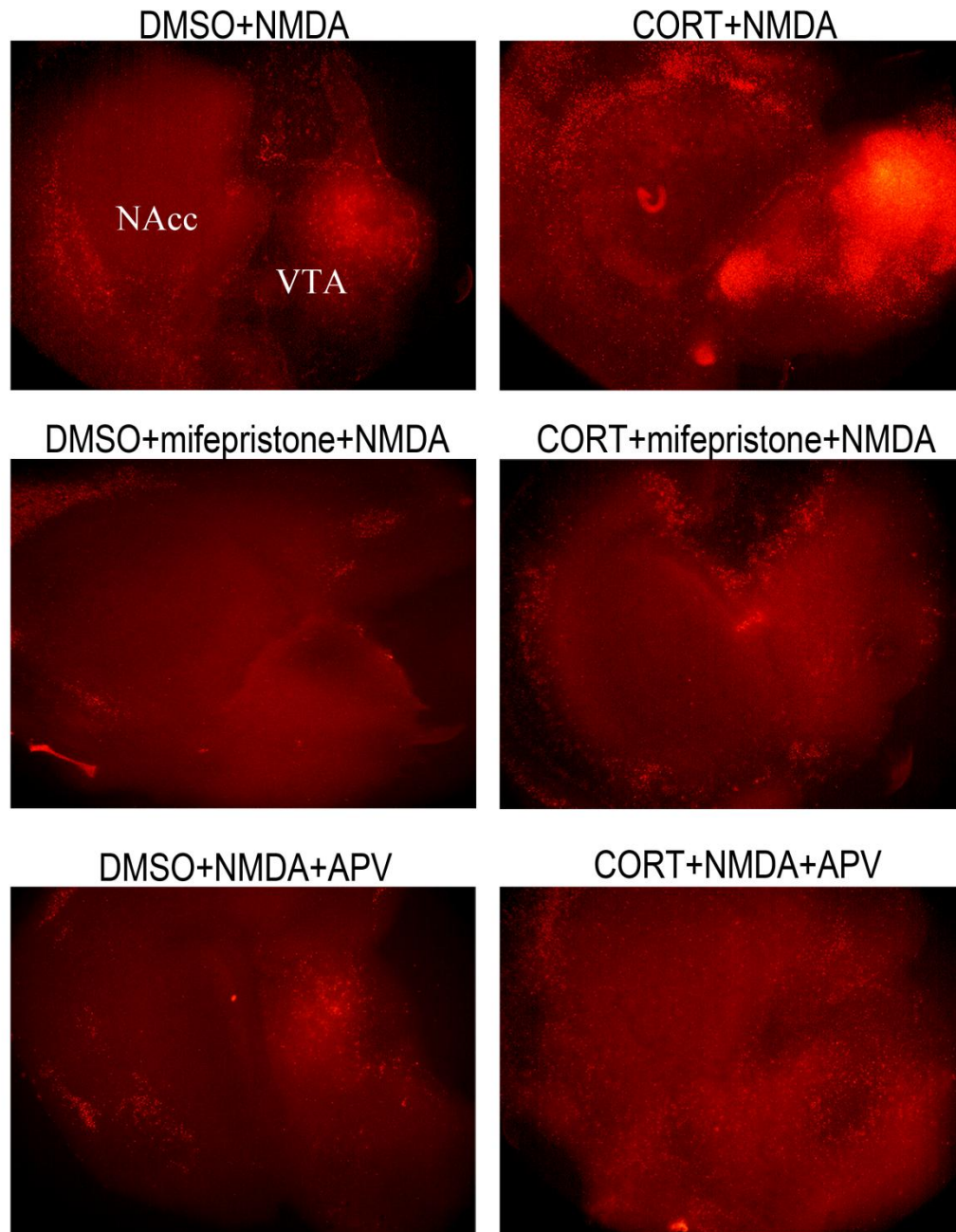


Figure 1.3. Representative images of PI uptake in co-cultures of the NAcc and VTA pretreated with either DMSO, CORT, DMSO + mifepristone, or CORT + mifepristone and exposed to NMDA or NMDA + APV for 24 hours.

PI Uptake	NAcc mean \pm SEM
DMSO	99.998 \pm 4.960
DMSO + mifepristone	116.247 \pm 6.567
DMSO + APV	98.734 \pm 4.197
DMSO + NMDA	90.558 \pm 4.313
DMSO + mifepristone + NMDA	*120.767 \pm 2.996
DMSO + NMDA + APV	112.903 \pm 6.842
CORT	95.542 \pm 3.716
CORT + mifepristone	118.572 \pm 6.377
CORT + APV	108.853 \pm 5.760
CORT + NMDA	96.700 \pm 4.771
CORT + mifepristone + NMDA	*134.002 \pm 3.754
CORT + NMDA + APV	99.648 \pm 11.851

Table 1.1. Effects of 5 day pre-treatment with either vehicle (DMSO), 1 μ M CORT, 10 μ M mifepristone, or CORT + mifepristone prior to 24 hour co-exposure to either vehicle/CORT/mifepristone + 200 μ M NMDA + 50 μ M APV on PI uptake within the nucleus accumbens (NAcc). Co-exposure to the glucocorticoid receptor antagonist mifepristone during the 5 day pre-treatment and subsequent NMDA exposure resulted in significant toxicity within the NAcc, while pre-treatment with CORT in the absence of mifepristone had no effect on NMDA-induced neuronal toxicity. * $p < 0.05$ vs. DMSO (vehicle) levels

Effects of CORT on NMDA-induced loss of NeuN Immunoreactivity (Figures 1.4 and 1.5; Table 1.2)

This set of experiments also sought to determine the effects of 5 days of previous CORT pre-exposure followed by 24 hours of co-exposure to CORT and NMDA and/or the competitive NMDA receptor antagonist APV on NeuN immunoreactivity. There was again no difference in immunoreactivity for NeuN between sexes within each treatment group, and male and female data were combined for further analysis. Thus, a one-way ANOVA for treatment was conducted within each region of interest. In the VTA, a significant main effect of treatment was observed (Figure 1.4; $F(11, 90)=3.172, p<0.001$). Regardless of acute treatment (NMDA/APV/mifepristone), vehicle-pretreated co-cultures showed no significant loss of NeuN immunoreactivity within the VTA (~90-100% vehicle control; Figure 1.4A). Additionally, exposure to continuous CORT (for a total of 6 days) in NMDA-naïve tissue produced no significant changes in the density of NeuN compared to vehicle treated co-cultures (~84% vehicle control; $p>0.05$; Figure 1.4B). In agreement with PI data described above, a significant loss of NeuN immunoreactivity was observed in the VTA after 5 days of pre-exposure to CORT, but not vehicle, followed by 24 hour exposure to CORT+NMDA (~80% vehicle control; $p<0.01$). Further, the CORT+NMDA-induced loss of NeuN was dependent on the NMDA receptor, as co-exposure to the NMDA receptor antagonist APV attenuated this loss (~93% vehicle control; $p<0.05$). In regards to the GR, pre-treatment with mifepristone during the 5 day exposure to CORT prior to the 24 hour co-exposure to NMDA significantly attenuated the CORT+NMDA-induced loss of NeuN (~95% vehicle control; $p<0.01$). Thus, within the VTA, chronic CORT pre-exposure followed by acute NMDA

exposure produced significant losses in NeuN immunoreactivity that was dependent on both NMDA receptor- and GR-activation. Representative images of these effects are presented in Figure 1.5. In contrast to the VTA, no significant main effects of treatment nor interactions were found in the NAcc region (table 1.2).

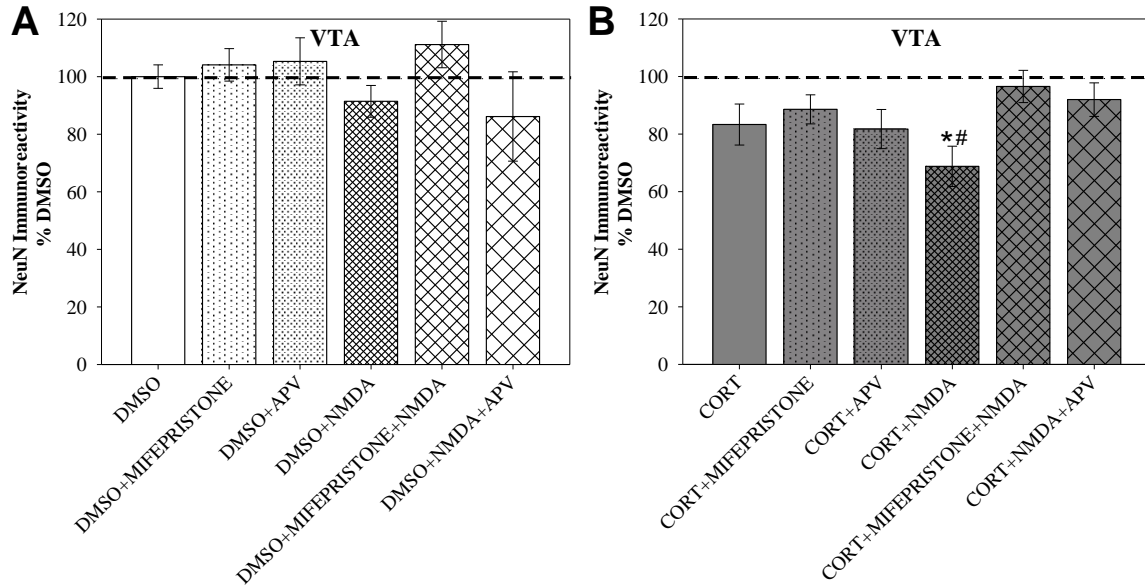


Figure 1.4. Effects of 5 day pre-treatment with either vehicle (DMSO; panel A), 1 μ M CORT (panel B), 10 μ M mifepristone, or CORT + mifepristone prior to 24 hour co-exposure to either vehicle/CORT/mifepristone along with 200 μ M NMDA + 50 μ M APV on immunoreactivity of the neuronal nuclear protein (NeuN) within the ventral tegmental area (VTA). Pre-treatment with CORT prior to NMDA exposure resulted in a significant loss of NeuN immunoreactivity, an effect which was not seen in vehicle pretreated cultures. Co-exposure to either the competitive NMDA receptor antagonist APV (during NMDA exposure) or to the glucocorticoid receptor antagonist mifepristone (during the 5 day CORT pre-treatment and subsequent NMDA exposure) significantly attenuated the ability of CORT to potentiate NMDA-induced toxicity. Dashed line represents DMSO-treated value. Data represented as percentage of DMSO-treated cultures (mean \pm SEM). * $p < 0.05$ vs. DMSO (vehicle) levels; # $p < 0.05$ vs. DMSO+NMDA, CORT+MIFEPRISTONE+NMDA, CORT+NMDA+APV.

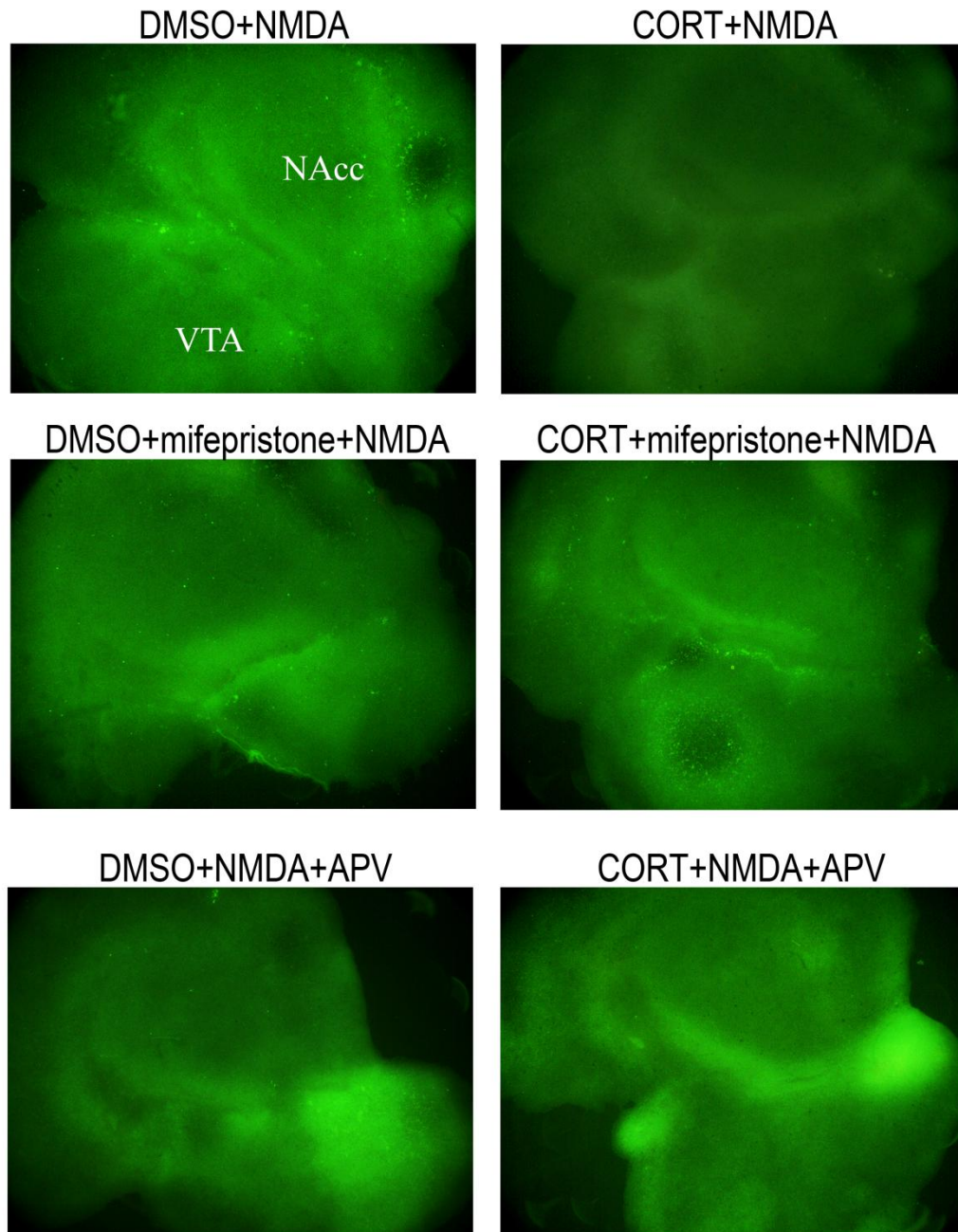


Figure 1.5. Representative images of NeuN immunoreactivity in co-cultures of the NAcc and VTA pretreated with either DMSO, CORT, DMSO + mifepristone, or CORT + mifepristone and exposed to NMDA or NMDA + APV for 24 hours.

NeuN Immunoreactivity	NAcc mean \pm SEM
DMSO	100.000 \pm 2.908
DMSO + mifepristone	92.565 \pm 6.041
DMSO + APV	98.543 \pm 5.006
DMSO + NMDA	102.821 \pm 1.592
DMSO + mifepristone + NMDA	89.571 \pm 4.678
DMSO + NMDA + APV	105.567 \pm 14.182
CORT	91.295 \pm 4.121
CORT + mifepristone	93.203 \pm 3.120
CORT + APV	89.989 \pm 5.601
CORT + NMDA	81.590 \pm 4.091
CORT + mifepristone + NMDA	95.285 \pm 3.055
CORT + NMDA + APV	92.746 \pm 4.959

Table 1.2. Effects of 5 day pre-treatment with either vehicle (DMSO), 1 μ M CORT, 10 μ M mifepristone, or CORT + mifepristone prior to 24 hour co-exposure to either vehicle/CORT/mifepristone + 200 μ M NMDA + 50 μ M APV on neuronal nuclear protein (NeuN) immunoreactivity within the nucleus accumbens (NAcc). No statistically significant changes were observed in immunoreactivity for NeuN.

Results – Experiment 2

Characterization of Triple Co-culture Model (Figure 2.1)

Several measures have also examined the validity of the triple co-culture model containing the VTA, NAcc, and PFC. Figure 2.1A (far top) shows a light microscopy picture of a control-treated co-culture, indicating the regions of interest and placement of each region so as to mimic the *in vivo* pathway. Further, immunoreactivity of MBP in control-treated co-cultures has been examined (Figure 2.1B; with the arrow indicating concentrated myelinated fibers at the junction of the co-cultures). Figure 2.1C (bottom) shows PI staining for neuronal toxicity following 24 hour exposure to 200 μ M NMDA in co-cultures, indicating the viability of all 3 regions of interest. These preliminary data indicate the neuronal viability and re-connectivity in triple slice co-cultures containing the VTA, NAcc, and PFC.

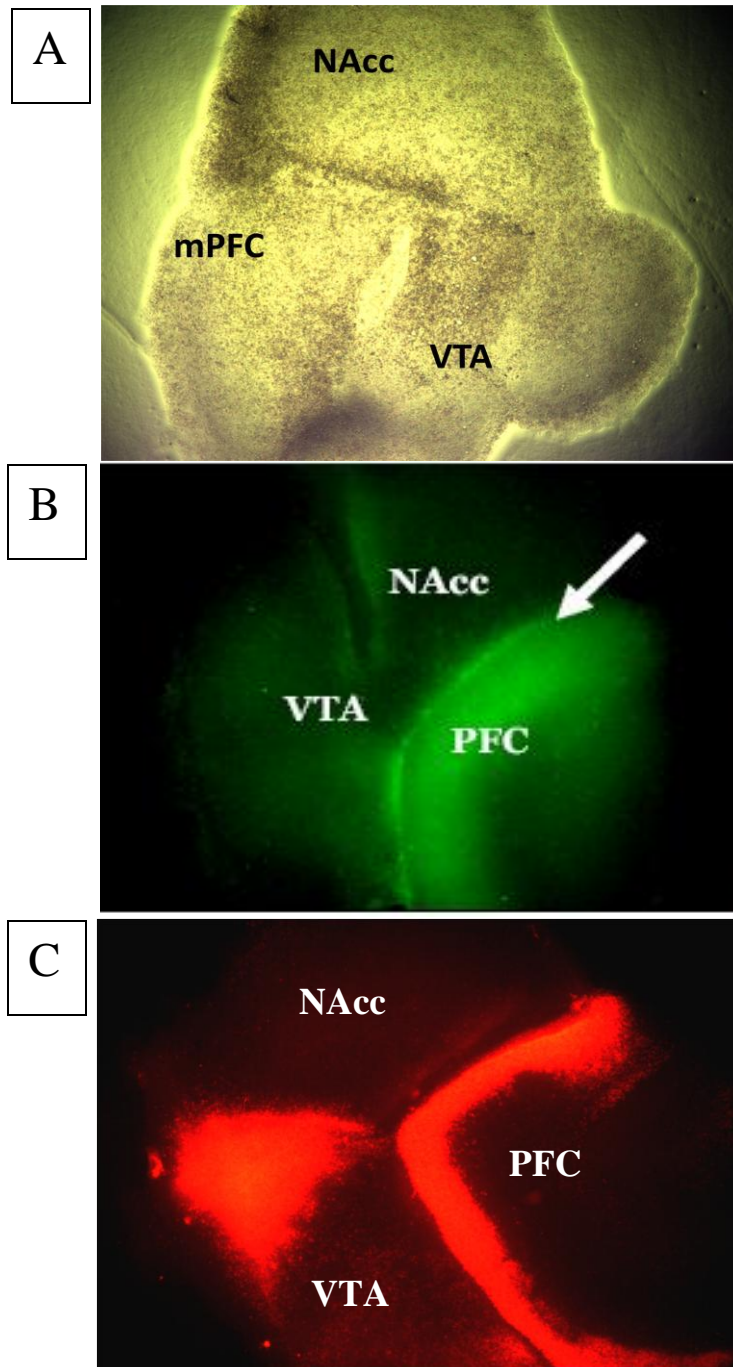


Figure 2.1. Triple slice co-cultures of VTA, NAcc, and mPFC. **Top (A):** Bright-field image of organotypic co-cultures containing the VTA, NAcc, and PFC showing the orientation of the co-culture, with the ventral striatum in contact with the VTA and the dorsal striatum in contact with the mPFC. **Middle (B):** Immunoreactivity of MBP in triple slice co-culture, with arrow indicating the junction of co-cultures. **Bottom (C):** image showing propidium iodide uptake following 24 hour exposure to NMDA (200 μ M), indicating NMDA-induced toxicity.

Effects of CORT on METH-induced PI Uptake (Figures 2.2 and 2.3)

This experiment was designed to assess the neurotoxic effects of 5 days of previous CORT pre-exposure followed by 24 hours of co-exposure to CORT and METH, and to investigate the potential role of the NMDA receptor in the observed cytotoxicity. There was no difference in toxicity between sexes within each treatment group, and male and female data were combined for further analysis. Thus, a one-way ANOVA for treatment was conducted within each region of interest. In the mPFC, a significant main effect of treatment was observed (Figure 2.2A; $F(7, 102)=5.325, p<0.001$). Five day pre-exposure to CORT followed by 24 hour co-exposure to CORT and METH resulted in significant toxicity in the mPFC compared to vehicle-pretreated co-cultures (~140% vehicle control; Fisher's LSD post-hoc, $p<0.001$). Further, neither CORT alone nor vehicle + METH produced significant toxicity in this region. The observed CORT and METH-induced toxicity within the mPFC was significantly attenuated by co-exposure to the NMDA receptor antagonist APV during the 24 hour co-exposure period ($p<0.01$). Within the NAcc, no significant main effects were observed (Figure 2.2B; $F(7, 102)=2.001, p=0.062$). In the VTA region, a significant main effect of treatment was observed (Figure 2.2C; $F(7, 102)=3.428, p<0.01$). Compared to vehicle-treated co-cultures, exposure to CORT for 5 days followed by 24 hour co-exposure to CORT and METH resulted in significant toxicity within the VTA (~135% vehicle control; $p<0.001$). The observed CORT + METH-induced toxicity within the VTA was significantly attenuated by co-exposure to the NMDA receptor antagonist APV during the 24 hour co-exposure period ($p<0.05$). However, significant toxicity was also observed following either 6 day exposure to CORT alone (~120% vehicle control; $p<0.01$) or 24 hour

exposure to METH in vehicle pre-treated co-cultures (~120% vehicle control; $p < 0.01$). Further, the observed CORT- or METH-induced toxicity was modestly attenuated by co-exposure to APV, although not to significant levels. Representative images of PI uptake in triple slice co-cultures exposed to CORT + METH are presented in Figure 2.3.

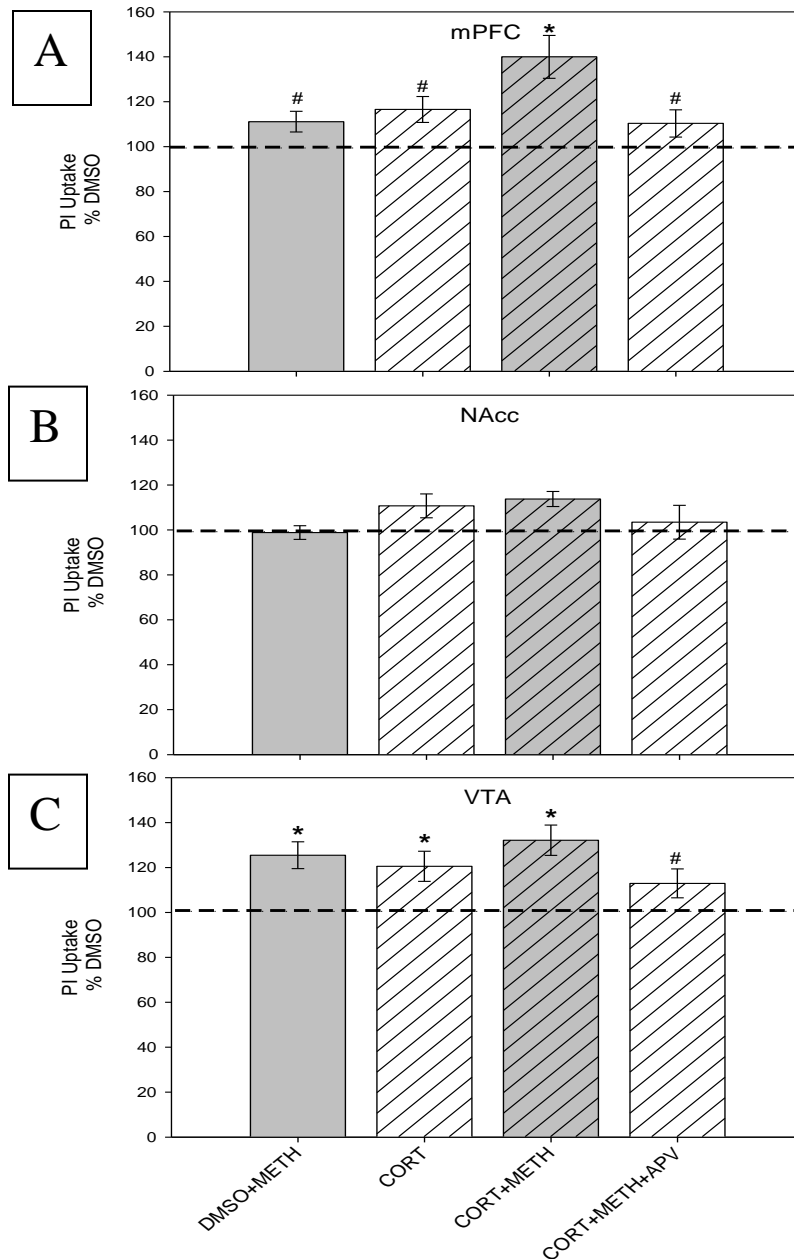


Figure 2.2. Effects of 5 day pre-treatment with either vehicle (DMSO) or 1 μ M CORT prior to 24 hour co-exposure to either vehicle/CORT along with 100 μ M METH + 50 μ M APV on PI uptake within the medial prefrontal cortex (**mPFC**; **A**), nucleus accumbens (**NAcc**; **B**) or ventral tegmental area (**VTA**; **C**). Pre-treatment with CORT and co-exposure to METH resulted in significant neurotoxicity in the mPFC, an effect which was not seen in vehicle pre-treated cultures. Co-exposure to the competitive NMDA receptor antagonist APV prevented the METH-induced cytotoxicity in CORT pre-treated cultures in the mPFC region. Dashed line represents DMSO-treated value. Data represented as percentage of DMSO-treated cultures (mean \pm SEM).* $p < 0.05$ vs. DMSO (vehicle) levels; # $p < 0.05$ vs. CORT + METH

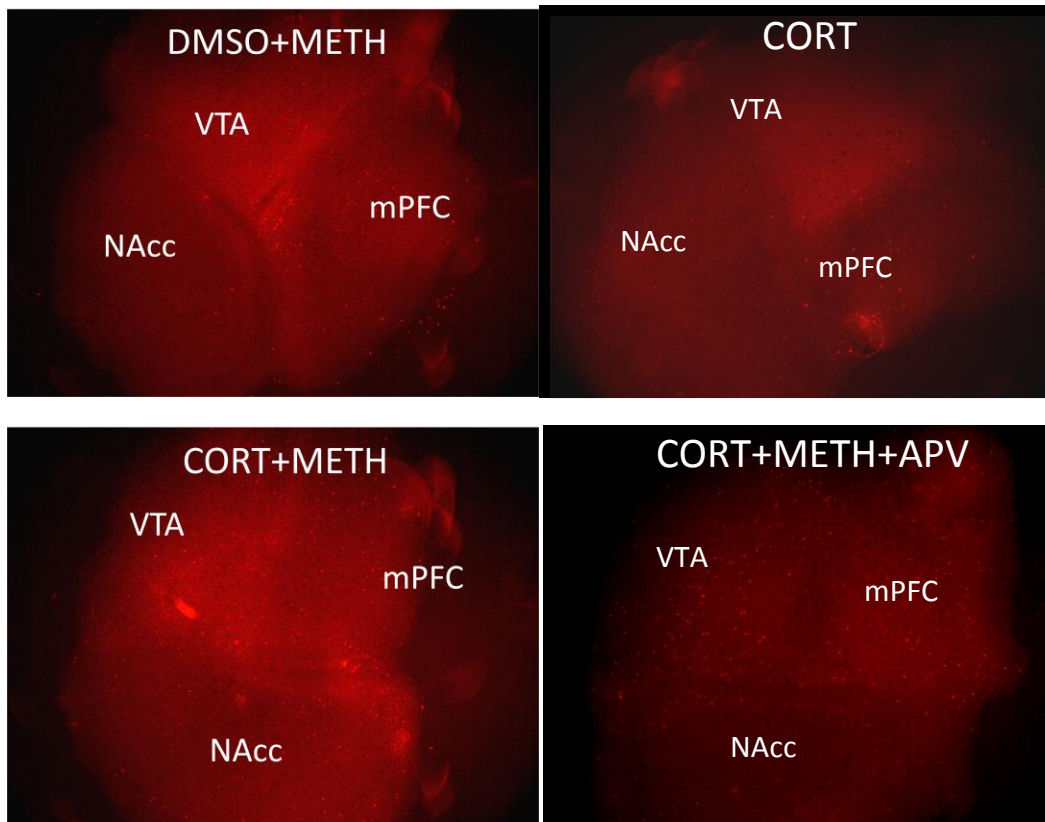


Figure 2.3. Representative images of PI uptake in co-cultures of the medial prefrontal cortex (mPFC), nucleus accumbens (NAcc) and ventral tegmental area (VTA) pretreated with either DMSO or CORT and co-exposed to METH (100 μ M) or METH + APV (50 μ M) for 24 hours.

Effects of CORT on METH-induced NeuN Immunoreactivity (Figures 2.4 and 2.5)

This experiment was designed to examine losses in immunoreactivity of the neuronal nuclear protein (NeuN) following 5 days of previous CORT pre-exposure followed by 24 hours of co-exposure to CORT and METH. There was no difference in NeuN immunoreactivity between sexes within each treatment group, and male and female data were combined for further analysis. Thus, a one-way ANOVA for treatment was conducted within each region of interest. Within the mPFC, no significant main effect of treatment was observed (Figure 2.4A; $F(7, 92)=0.643, p>0.05$). Similarly, in the NAcc as well as the VTA, no significant main effects were observed (Figure 2.4B and 2.4C, respectively; $F(7, 92)=0.848, p>0.05$ for NAcc; $F(7, 92)=0.801, p>0.05$ for VTA). Representative images of NeuN immunoreactivity in triple slice co-cultures exposed to CORT + METH are presented in Figure 2.5.

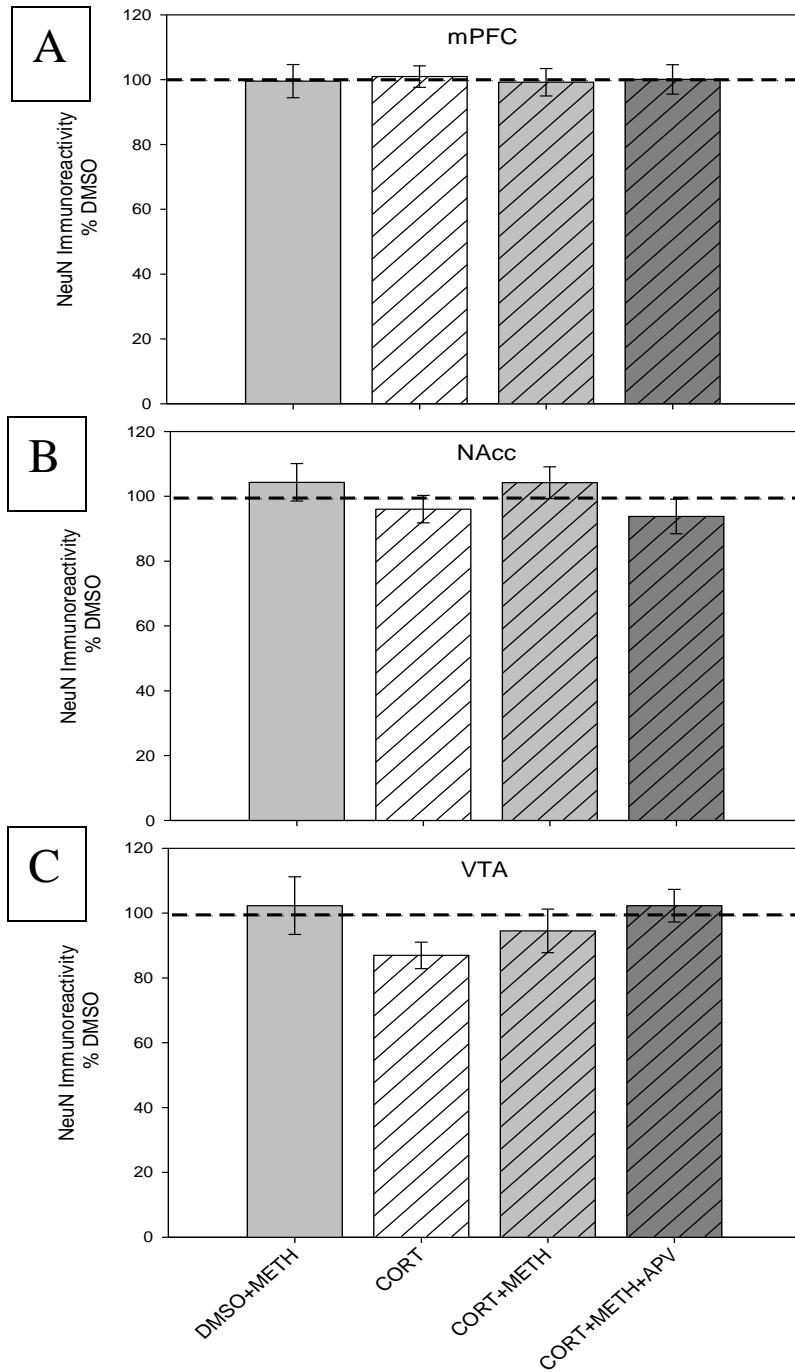


Figure 2.4. Effects of 5 day pre-treatment with either vehicle (DMSO) or 1 μ M CORT prior to 24 hour co-exposure to either vehicle/CORT along with 100 μ M METH + 50 μ M APV on immunoreactivity of neuronal nuclear protein (NeuN) within the medial prefrontal cortex (**mPFC**; **A**), nucleus accumbens (**NAcc**; **B**) or ventral tegmental area (**VTA**; **C**). Pre-treatment with CORT and co-exposure to METH did not result in significant losses in NeuN immunoreactivity in any of the regions examined.

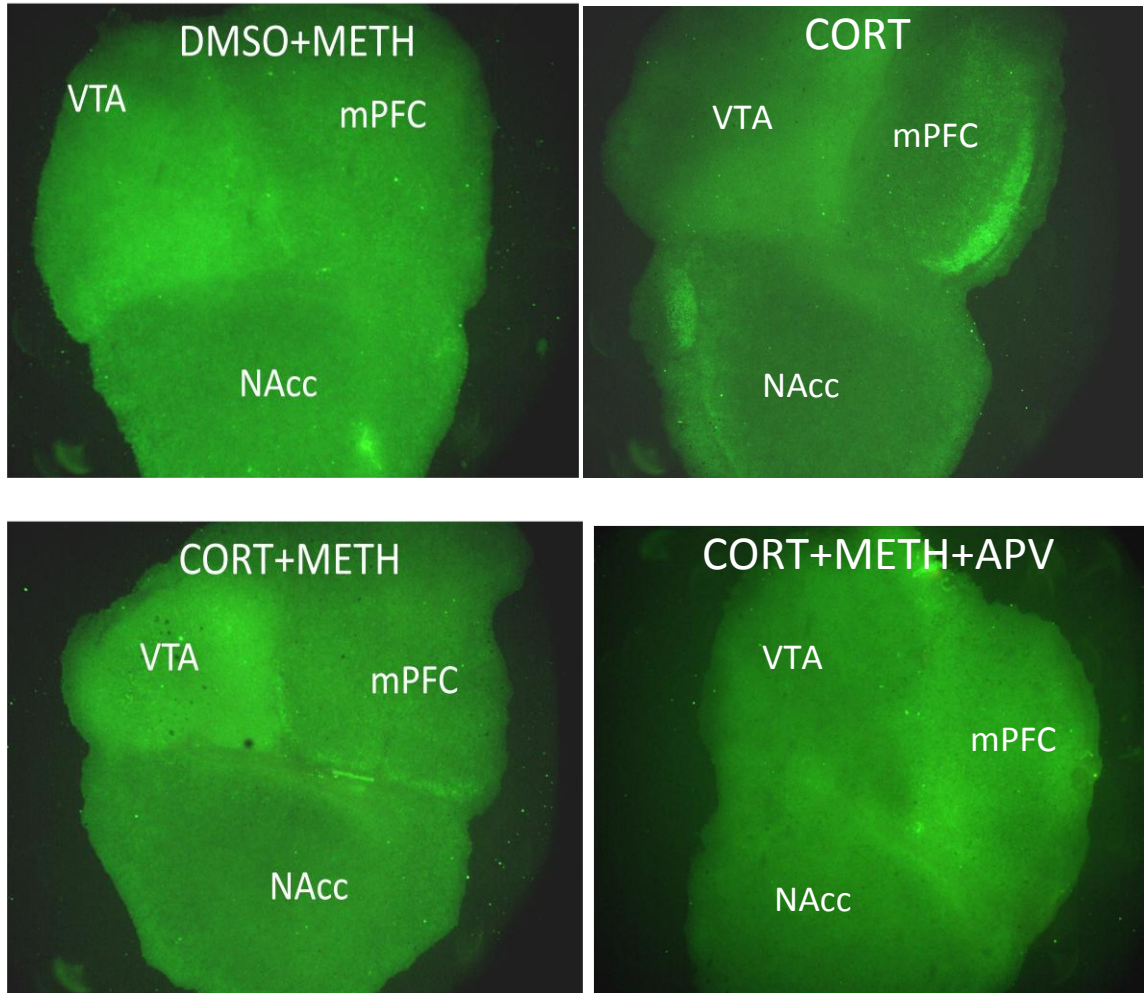


Figure 2.5. Representative images of immunoreactivity of neuronal nuclear protein (NeuN) in co-cultures of the medial prefrontal cortex (mPFC), nucleus accumbens (NAcc) and ventral tegmental area (VTA) pretreated with either DMSO or CORT and co-exposed to METH (100 μ M) or METH + APV (50 μ M) for 24 hours. No statistically significant group differences were observed.

Effects of CORT and METH on NR1 subunit density (Figures 2.6 and 2.7)

A second set of experiments was conducted to assess the effects of 5 days of CORT pre-exposure followed by 24 hours of co-exposure to CORT and METH on the density of the obligatory NMDA receptor subunit 1 in triple slice co-cultures. A one-way ANOVA for treatment revealed no significant main effect of treatment (Figure 2.6; $F(3, 16)=1.946, p>0.05$). Though not statistically significant, it is important to note that slight losses in NR1 density were observed in all METH-treated co-cultures (~80% vehicle treated control). It must also be noted that although region-specific effects were observed in experiment 1, western blot analysis was conducted in homogenized tissue containing all three regions of interest. A representative image of a western blot analysis for NMDA NR1 subunit density in triple slice co-cultures exposed to CORT + METH is presented in Figure 2.7.

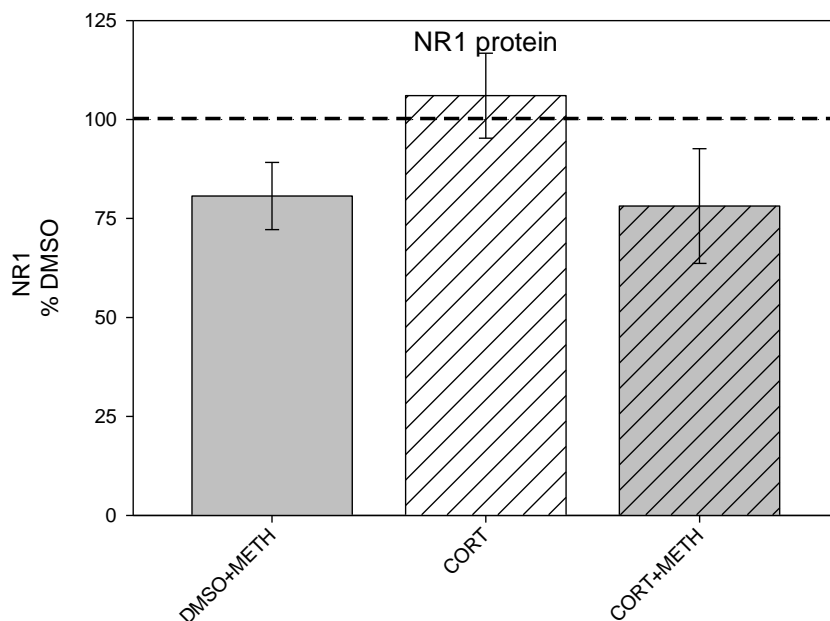
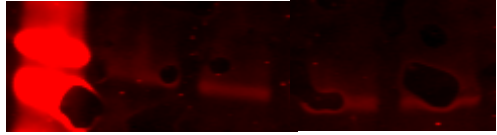


Figure 2.6. Effects of 5 day pre-treatment with either vehicle (DMSO) or 1 μ M CORT prior to 24 hour co-exposure to either vehicle/CORT along with 100 μ M METH on NR1 subunit protein density. No statistically significant changes in NR1 western blot analysis were observed following the 5 day vehicle/CORT pre-treatment + 24 hour co-exposure to METH.



DMSO	CORT	DMSO+	CORT+
		METH	METH

Figure 2.7. Representative image of western blot immunoblotting for the NMDA receptor NR1 subunit.

Effects of CORT and METH on NR2B subunit density (Figures 2.8 and 2.9)

The second set of experiments also assessed the effects 5 days of CORT pre-exposure followed by 24 hours of co-exposure to CORT and METH on the density of the polyamine sensitive NMDA receptor subunit 2B in triple slice co-cultures. A one-way ANOVA revealed a significant main effect of treatment on NR2B subunit density (Figure 2.8; $F(3, 16)=4.065$, $p<0.05$). Similar to the losses observed in NR1 density, significant losses in NR2B density were observed in co-cultures pre-treated with either vehicle or CORT and co-exposed to vehicle/CORT and METH for 24 hours as compared to vehicle-treated co-culture samples (~70% vehicle treated control, $p<0.05$). Exposure to CORT for 6 days in the absence of METH did not significantly alter NR2B subunit density. A representative image of a western blot analysis for NMDA NR2B subunit density in triple slice co-cultures exposed to CORT + METH is presented in Figure 2.9.

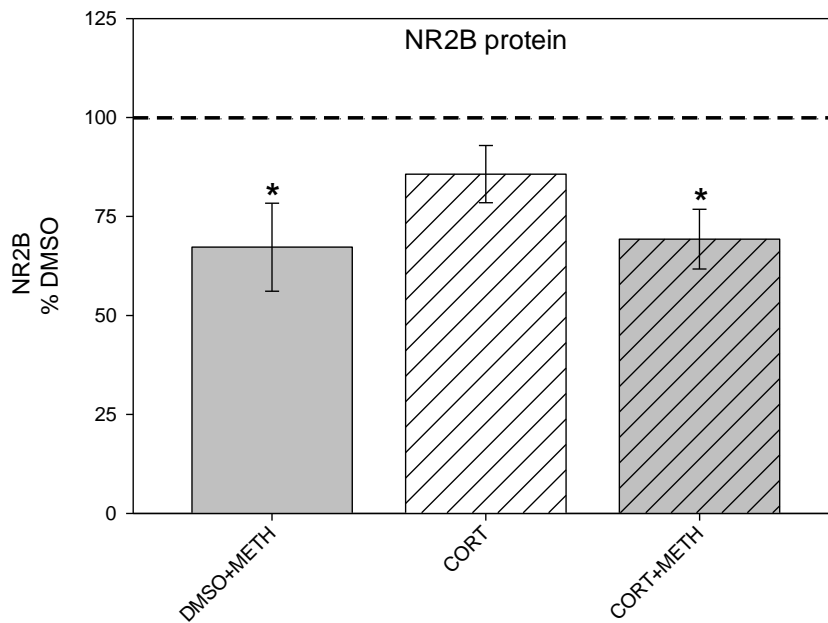


Figure 2.8. Effects of 5 day pre-treatment with either vehicle (DMSO) or 1 μ M CORT prior to 24 hour co-exposure to either vehicle/CORT along with 100 μ M METH on NMDA receptor NR2B subunit protein density. Exposure to METH for 24 hours significantly reduced NR2B subunit density as measured by western blot analysis regardless of pretreatment (DMSO/CORT). Dashed line represents DMSO-treated value. Data represented as percentage of DMSO-treated cultures (mean \pm SEM). * $p < 0.05$ vs. DMSO (vehicle) levels

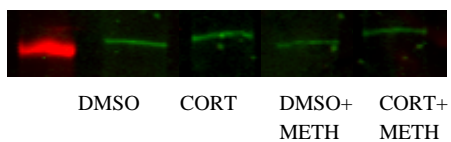


Figure 2.9. Representative image of western blot immunoreactivity for the NMDA receptor NR2B subunit.

Effects of CORT and METH on HPLC analysis of extracellular dopamine (Figures 2.10 and 2.11)

Experiment 2C was designed to assess the effects of 5 days of CORT pre-exposure followed by 24 hours of co-exposure to CORT and METH on extracellular DA and GLUT levels, and to examine the potential role of the NMDA receptor in any CORT-induced augmentation of DA and GLUT levels in response to acute METH. Experiment 2C focused on extracellular DA levels in triple slice co-cultures co-exposed to CORT and METH for 24 hours in the presence of the NMDA receptor antagonist APV. A one-way ANOVA revealed no significant main effect of treatment on DA HPLC (Figure 2.10; $F(7, 63)=1.075, p=0.390$). However, it is important to note that METH-treated cultures (both vehicle- and CORT-pretreated) had extracellular dopamine levels equal to approximately 80-85% of vehicle control values, suggesting the possibility of slight dopaminergic toxicity. A representative image of a chromatograph for DA HPLC analysis using ACSF samples collected from triple slice co-cultures exposed to CORT + METH is presented in Figure 2.11.

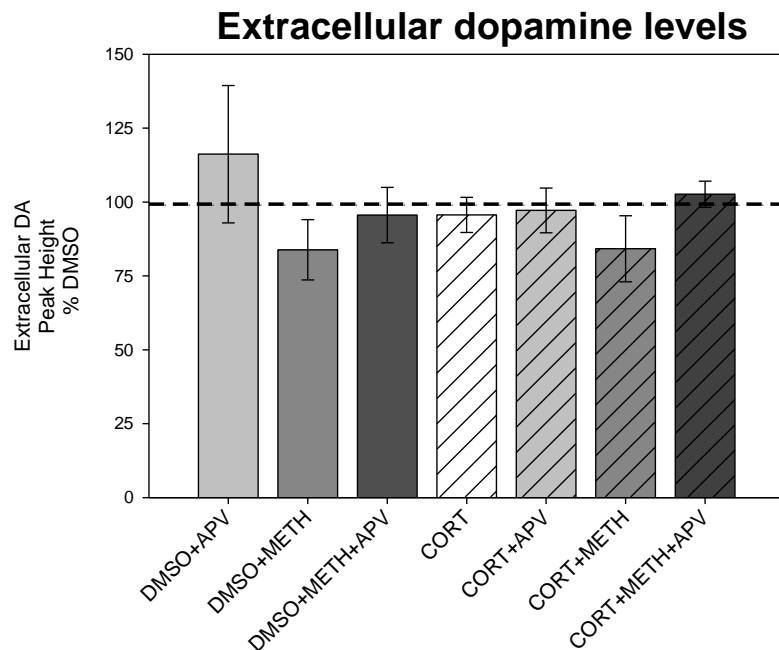


Figure 2.10. Effects of 5 day pre-treatment with either vehicle (DMSO) or 1 μ M CORT prior to co-exposure to either vehicle/CORT along with 100 μ M METH on extracellular dopamine levels in artificial cerebrospinal fluid samples collected 45 minutes post-METH. There were no statistically significant changes in extracellular dopamine following CORT and/or METH exposure. Dashed line represents DMSO-treated value. Data represented as percentage of DMSO-treated cultures (mean \pm SEM).

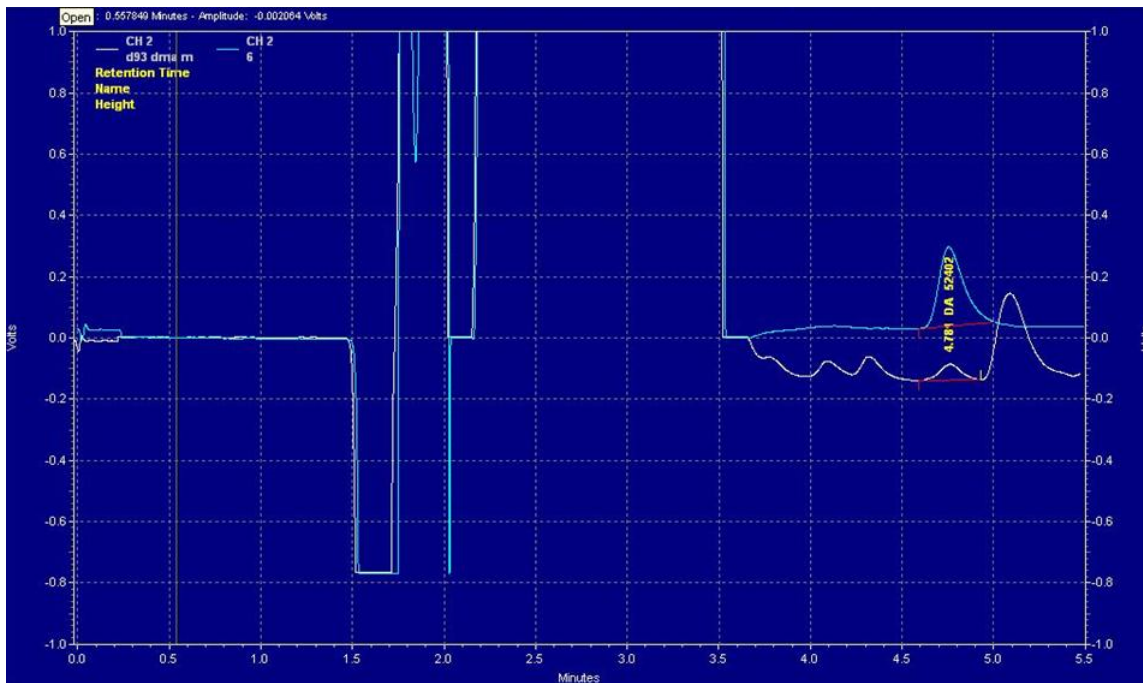


Figure 2.11. Representative image of DA HPLC chromatograph using ACSF samples collected from triple slice co-cultures exposed to CORT + METH, showing a vehicle-treated control (white) and a 6 nM DA standard (blue).

Effects of CORT and METH on HPLC analysis of extracellular glutamate (Figures 2.12 and 2.13)

Experiment 2C was designed to assess the effects of 5 days of CORT pre-exposure followed by 24 hours of co-exposure to CORT and METH on extracellular DA and GLUT levels, and to examine the potential role of the NMDA receptor in any CORT-induced augmentation of DA and GLUT levels in response to acute METH. Experiment 2C focused on extracellular GLUT levels in triple slice co-cultures co-exposed to CORT and METH for 24 hours in the presence of the NMDA receptor antagonist APV. A one-way ANOVA revealed a significant main effect of treatment on GLUT HPLC (Figure 2.12; $F(7, 67)=60.535$, $p<0.001$). Five day pre-exposure to CORT followed by 45 minute co-exposure to CORT and METH resulted in a significant decrease in extracellular GLUT levels compared to vehicle-pretreated co-cultures (~75% vehicle control; Fisher's LSD post-hoc, $p<0.001$). Further, neither CORT alone nor vehicle + METH produced any significant changes in extracellular GLUT levels compared to vehicle controls. The decrease in extracellular GLUT levels following CORT + METH exposure was significantly different than that seen with CORT ($p<0.01$). The observed CORT and METH-induced decrease in extracellular GLUT was significantly reversed by co-exposure to the NMDA receptor antagonist APV during the 45 minute co-exposure period ($p<0.001$). In fact, all APV-treated cultures had significantly higher levels of extracellular GLUT compared to vehicle controls (~145-165% vehicle control; $p<0.001$). The combination of CORT + METH + APV was not significantly lower than the combination of vehicle (DMSO) + METH + APV ($p=0.196$), but was significantly lower compared to CORT + APV or vehicle + APV ($p<0.01$). A representative image of a

chromatograph for GLUT HPLC analysis using ACSF samples collected from triple slice co-cultures exposed to CORT + METH is presented in Figure 2.13.

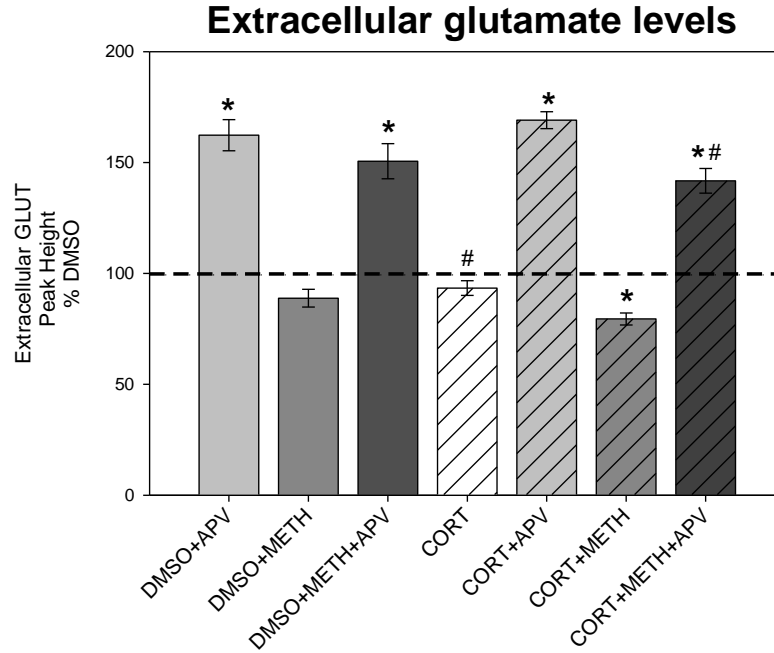


Figure 2.12. Effects of 5 day pre-treatment with either vehicle (DMSO) or 1 μ M CORT prior to co-exposure to either vehicle/CORT along with 100 μ M METH on extracellular glutamate levels in artificial cerebrospinal fluid samples collected 45 minutes post-METH. Exposure to METH in CORT-pretreated cultures resulted in a significant loss of extracellular glutamate compared to vehicle-pretreated cultures; this effect was significantly altered by co-exposure to the NMDA receptor antagonist APV during METH. Dashed line represents DMSO-treated value. Data represented as percentage of DMSO-treated cultures (mean \pm SEM). * $p < 0.05$ vs. DMSO (vehicle) levels; # $p < 0.05$ vs. CORT + METH

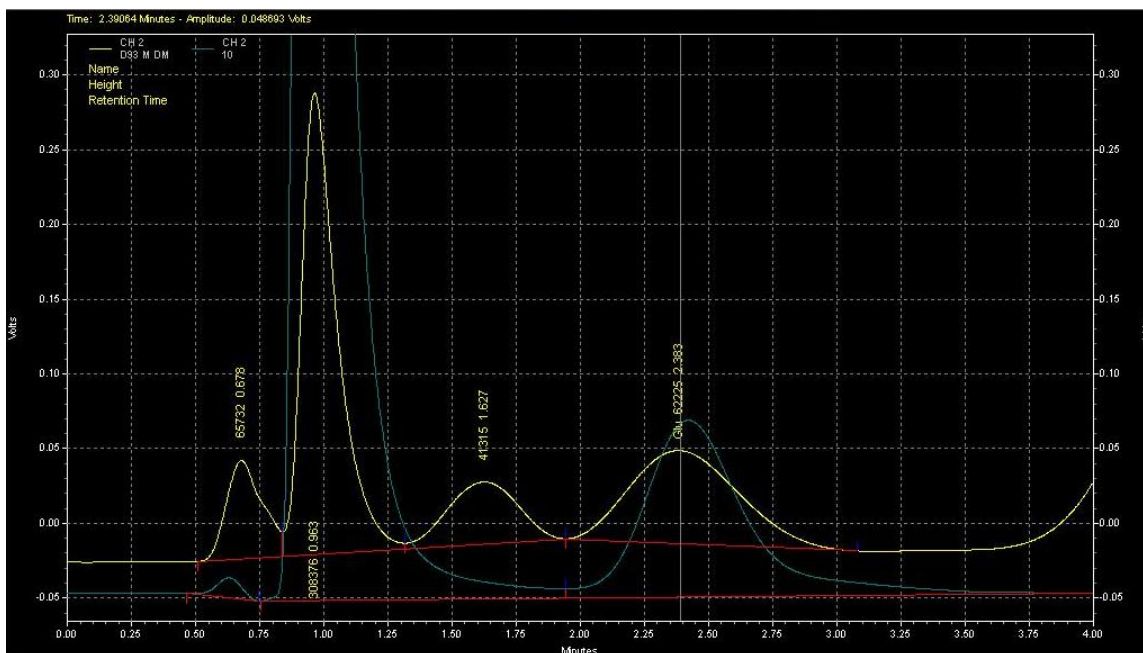


Figure 2.13. Representative image of GLUT HPLC chromatograph using ACSF samples collected from triple slice co-cultures exposed to CORT + METH, showing a vehicle-treated control (yellow) and a 10 nM GLUT standard (green).

CHAPTER 4

DISCUSSION

Enhanced Toxicity Following CORT and NMDA Exposure

Previous findings have suggested that 24 hour CORT co-exposure can significantly exacerbate the toxicity associated with the overactivation of the NMDA receptor in the hippocampus in a NMDA receptor-dependent manner (Mulholland et al., 2006). Notably, these earlier findings reported that this effect was independent of GR activation and was cyclohexamide insensitive. Further studies have investigated the role of the MR and GR in the ability of chronic CORT exposure to potentiate NMDA-induced cytotoxicity. Mulholland et al. (2004b) found that chronic co-exposure to CORT in the presence of the GR antagonist mifepristone, but not the MR antagonist spironolactone, attenuated subsequent NMDA receptor-mediated toxicity. The present studies (experiment 1) extend those by investigating the potential CORT-induced changes in response to an excitotoxin in regions critical to reward. Results from experiment 1 demonstrate that chronic pre-treatment with CORT exacerbated NMDA receptor-mediated neurotoxicity in the VTA, but not the NAcc. Further, this effect was dependent upon the NMDA receptor, as co-exposure to the NMDA receptor antagonist APV during NMDA exposure prevented the CORT-induced potentiation. Additional studies also demonstrated that the ability of CORT to potentiate NMDA-induced neurotoxicity within the VTA was dependent upon GR activation, as co-exposure to the GR antagonist mifepristone during CORT exposure significantly attenuated the subsequent NMDA receptor-mediated toxicity. Although previous studies have suggested a potential role for

plasma membrane-bound CORT receptors (Xiao, Feng, & Chen, 2010), the present data demonstrate the importance of intracellular GR binding in mediating the effects of CORT in this reward pathway. The ability of CORT to potentiate subsequent NMDA receptor-mediated excitotoxic insults within areas of the brain key to drug reward and reinforcement may be of crucial importance as several drugs of abuse, such as METH, cocaine, and withdrawal from ethanol, are known to produce increases in extracellular GLUT release within the striatum resulting in neurotoxicity (Abekawa, et al., 1994; Rossetti & Carboni, 1995; Rossetti, Carboni, & Fadda, 1999; Wakabayashi & Kiyatkin, 2012). Taken together, these findings suggest that chronic elevations in CORT may sensitize ventral tegmental neurons to subsequent excitatory effects of drugs of abuse and that NMDA receptors may serve as a viable therapeutic target for individuals with chronically elevated levels of cortisol (i.e. in individuals with stress disorders such as PTSD).

Regional Differences in CORT + NMDA-induced Toxicity

It is interesting to note that CORT-induced potentiation of NMDA receptor-mediated neurotoxicity only occurred in the VTA and not in the NAcc. This does not likely reflect differences in NMDA receptor binding as previous reports have found evidence for a higher density of NMDA receptor binding sites in the NAcc compared to a low relative density in the VTA (Albin et al., 1992). Additional studies have examined the distribution of subunit-specific antibodies for the NMDA receptor and also found no significant differences in the distribution of NR2A nor NR2B within the NAcc and VTA (Wang et al., 1995), although the polyamine sensitive NR2B subunit appears to be the most prevalent subunit in mesencephalon cultures, as compared to the NR2A, NR2C, and

NR2D subunits (Allgaier, Scheibler, Muller, Feuerstein, & Illes, 1999). Notably, as the NR2B subunit is sensitive to polyamine synthesis, CORT has also been shown to dose-dependently increase polyamine expression within the hippocampus (Ientile, De Luca, Di Giorgio, & Macaione, 1988), thus providing a possible mechanism for the regional specificity for CORT effects within the VTA. One alternative hypothesis is that there is a differential distribution of GRs between the NAcc and VTA. Although the distribution of GRs is often thought to be ubiquitous throughout much of the rodent brain, results from previous studies have been mixed regarding distribution within the NAcc and VTA. For example, Aronsson et al. (1988) found moderate levels of GR mRNA in the VTA compared to weak GR mRNA in the NAcc. Most studies, however, have found approximately equal distribution of GRs via immunohistochemistry and/or in situ hybridization within the striatum and midbrain regions (Ahima & Harlan, 1990; Morimoto, Morita, Ozawa, Yokoyama, & Kawata, 1996). Additionally, several studies have noted strong immunoreactivity for the GR among dopaminergic neurons, especially those originating in the VTA (Barik et al., 2010; Diaz, Sokoloff, & Fuxe, 1997; Harfstrand, et al., 1986).

Glucocorticoid Receptor-Dependent Toxicity Following CORT and NMDA Co-exposure

The results of the current study are in agreement with a large body of literature implicating the importance of the GR in the CORT-induced potentiation of a future insult (Alexander, DeVries, Kigerl, Dahlman, & Popovich, 2009; Mulholland, et al., 2004a, 2004b), although the exact mechanism by which CORT may enhance neuronal vulnerability to future insults is not yet well defined. Research suggests that long-term exposure to high levels of circulating glucocorticoids may increase the expression and/or

function of the NMDA receptor subunits (Karst & Joels, 2003; Prendergast & Mulholland, 2012; Weiland, et al., 1997). In addition, high concentrations of CORT have been shown to result in increased intracellular levels of Ca^{2+} (Karst, Wadman, & Joels, 1994; Kerr, Campbell, Thibault, & Landfield, 1992) as well as induce the release of GLUT and other excitatory amino acids (Moghaddam, Bolinao, Stein-Behrens, & Sapolsky, 1994; Stein-Behrens et al., 1992; Stein-Behrens, Lin, & Sapolsky, 1994). Notably, the use of mifepristone in the current study does not preclude the role of progesterone receptors in the observed results as mifepristone, in addition to its action at the GR, is a progesterone receptor antagonist. Future experiments with a more selective GR antagonist are necessary to rule out the possibility that progesterone receptor antagonism protects against CORT and NMDA-induced toxicity. Although other mechanisms cannot be ruled out, the results of the current study in conjunction with several previous studies suggest that chronic CORT may lead to increased neuronal vulnerability to future excitotoxic insults via increased NMDA receptor expression and/or function.

Stress Exacerbates METH-induced Toxicity

Many *in vivo* studies have noted toxicity following high doses of METH, an effect which is further perturbed by chronically stressing the animal prior to METH administration. In particular, in a study by Nash and Yamamoto (1992) rats were given 3 injections of 7.5 mg/kg (i.p.) of METH every 2 hours during microdialysis. Repeated METH administration resulted in increased extracellular GLUT concentrations in the striatum compared to saline or ecstasy (MDMA) administration. In contrast, DA concentrations within the striatum were also measured using microdialysis and were

increased following each injection of both METH and MDMA. Seven days after the repeated METH or MDMA administration, DA content within the striatum was measured; repeated METH resulted in decreased DA content compared to vehicle, whereas repeated MDMA administration had no effect on DA content in the striatum. Taken together, these results suggest that long term METH-induced dopaminergic toxicity is, in part, mediated by GLUT. Previous exposure to chronic stress can exacerbate METH-induced increases in striatal GLUT and decreases of striatal DA content. Recently, Tata and Yamamoto (2008) found that animals previously exposed to a 10-day chronic unpredictable stress paradigm showed enhanced striatal extracellular GLUT concentrations in response to METH. Similarly, previous exposure to chronic stress exacerbated the METH-induced decrease in DA content within the striatum. Further, only animals in the chronically stressed condition and subsequently given the METH regimen showed increases in spectrin breakdown product, which may be indicative of NMDA receptor-mediated activation of calpains and related cysteine proteases. As these *in vivo* studies illustrate the enhanced vulnerability to METH-induced toxicity resulting from stress (and probable elevations in glucocorticoids), the current studies examined the effect of CORT pre-exposure on toxicity following acute METH exposure using an *in vitro* model of the mesocorticolimbic reward pathway. In kind with previous studies, we hypothesized that co-cultures exposed to CORT and METH would be most vulnerable to damage compared to co-cultures exposed to either CORT or METH independently. Additionally, we hypothesized that the damage caused by the combination of CORT and METH would be NMDA receptor-dependent and a result of neuroadaptations in the obligatory NR1 and polyamine sensitive NR2B subunits

of the glutamatergic NMDA receptor. A final set of experiments investigated the ability of antagonism of the NMDA receptor to prevent the CORT-induced potentiation of METH-evoked DA and GLUT overflow.

Toxicity Following CORT and Acute METH Co-exposure

The current data demonstrate that 5 day pre-exposure to CORT followed by 24 hour co-exposure to CORT and METH results in significant toxicity within the mPFC region, with CORT+METH co-exposure resulting in increased PI uptake by approximately 40-50% above vehicle-treated values. Further, the toxicity observed following CORT+METH co-exposure was significantly elevated compared to toxicity observed in cultures exposed to either CORT or METH independently. The current studies also demonstrate the importance of the NMDA receptor in CORT+METH-induced toxicity, as co-exposure to the NMDA receptor antagonist APV during CORT+METH exposure attenuated the observed toxicity. No significant toxicity was observed in the NAcc region following CORT, METH, or CORT+METH exposure. In contrast, within the VTA, significant toxicity was observed following either CORT or METH exposure (~20% above vehicle control values) while co-exposure to CORT+METH resulted in toxicity slightly greater than seen with either CORT or METH alone (~40% above vehicle-treated cultures). The additive toxic effects of CORT and METH in the VTA were significantly attenuated by co-exposure to APV during CORT+METH co-exposure.

In contrast to the current dataset which did not identify cytotoxicity in the striatum, several rodent studies have shown enhanced toxicity in the striatum following

stress or CORT and METH exposure (Matuszewich & Yamamoto, 2004a, 2004b; Quinton & Yamamoto, 2007; Raudensky & Yamamoto, 2007b; Tata, Raudensky, & Yamamoto, 2007; Tata & Yamamoto, 2008). However, it must be noted that significant methodological differences exist between the aforementioned *in vivo* studies and the current set of experiments. For example, many *in vivo* studies have used the chronic unpredictable stressor paradigm to induce stressful conditions and a resulting rise in CORT levels whereas the current study maintained a steady level of CORT prior to METH treatment in the culture system. Notably, *in vivo* high-dose METH is also known to produce hyperthermia (Ali, Newport, & Slikker, 1996; Miller & O'Callaghan, 2003), while the current *in vitro* studies maintained all cultures at 37°C. Further, many *in vivo* studies have focused on the long-term METH-induced dopaminergic terminal toxicity observed within the striatum (Matuszewich & Yamamoto, 2004a, 2004b; Quinton & Yamamoto, 2007; Raudensky & Yamamoto, 2007b; Tata, et al., 2007; Tata & Yamamoto, 2008); the current results suggest that the mPFC and VTA may be preferentially vulnerable to the short-term toxic effects of METH. Recently, Kelly and colleagues (2012) showed that mice pre-treated with CORT exhibited an enhanced striatal neuroinflammatory and neurotoxic response to an acute high-dose METH regimen; consistent with the current data, however, chronic CORT pre-treatment also sensitized the frontal cortex and the hippocampus to the neurotoxic effects associated with METH. Collectively, these data reveal the enhanced toxicity associated with the combination of CORT and METH, although discrepancies remain as to regional vulnerability.

NMDA Receptor Antagonism Protects Against CORT + METH-induced Toxicity

Prior literature has been divided as to the protection of METH-induced damage provided by antagonism of the NMDA receptor. Staszewski and Yamamoto (2006) found that repeated high-dose METH caused an increase in calpain-mediated spectrin proteolysis in the rodent striatum up to seven days following repeated METH administration; the METH-induced increase in spectrin proteolysis was attenuated by AMPA, but not NMDA, receptor antagonism. Further, neither AMPA nor NMDA receptor antagonists blocked METH-induced reductions in striatal DAT content (Staszewski & Yamamoto, 2006). Previous studies have demonstrated the neuroprotective effects of NMDA receptor antagonists against METH-induced toxicity (Sonsalla, 1995; Sonsalla, et al., 1991); however, *in vivo* studies have also revealed the importance of hyperthermia in METH-induced toxicity and the protection of such hyperthermia afforded by NMDA receptor antagonism (Miller & O'Callaghan, 2003). The current results suggest that, independent of hyperthermia, NMDA receptor antagonists may be useful in attenuating toxicity associated with CORT and/or METH.

Regional Differences in CORT + METH-induced Toxicity

The current study demonstrated region-specific effects of the combination of CORT and METH, including enhanced cytotoxicity following chronic CORT and acute METH exposure only in the mPFC. Although the current study did not examine regional differences in receptor distribution, the frontal cortex and mPFC has been reported to contain greater levels of the NMDA receptor compared to either the NAcc or VTA (Sakurai, Cha, Penney, & Young, 1991; Takita, Yokoi, & Mizuno, 1997). Additional

reports have suggested a high expression of mRNA for the NR2B subunit as well as high NR2B protein levels in the PFC compared to the NAcc (Loftis & Janowsky, 2003; Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994; Wenzel, Fritschy, Mohler, & Benke, 1997). Further, the vulnerability of the mPFC to CORT and METH-induced toxicity may be related to distribution of the GR. Previous studies in non-human primates showed that the PFC contains surprisingly high levels of GR mRNA (Sanchez, Young, Plotsky, & Insel, 2000). Consistent with the theory of regional differences in GR protein, recent work has shown that GR antagonism by RU-43044 prevents METH-induced increases in extracellular prefrontal DA levels whereas RU-43044 had no effect on extracellular DA levels measured in the NAcc shell (Ago et al., 2009). Further, prior stress synergistically enhanced lipopolysaccharide-induced neuroinflammation in the mPFC while GR antagonism by RU-486 protected against the combination of stress and lipopolysaccharide (de Pablos et al., 2006). Together, these results suggest that the mPFC may be particularly vulnerable to the combination of CORT and METH as a result of increased expression of GR or NMDA receptors.

Toxic Effects of METH in the VTA

The present studies revealed a region-specific vulnerability of the VTA to the toxic effects associated with exposure to either CORT or METH independently. Contradictory to the current results, cresyl violet staining of Nissl bodies in the VTA and substantia nigra revealed no effect of repeated METH (Ricaurte, Guillery, Seiden, Schuster, & Moore, 1982). Similarly, adult rodents exposed to three administrations of METH (10 mg/kg every 2 hours) had no significant changes in DA and serotonin levels in the VTA, substantia nigra, PFC, or hippocampus while METH significantly depleted

striatal DA and serotonin by 65% and 79%, respectively (Bisagno, Ferguson, & Luine, 2002). Keller and colleagues (2011) found a significant increase in DA tissue content in the VTA, as well as an accompanying significant decrease in the NAcc, 48 hours following escalating (0.1 – 4 mg/kg/day) or binge (3 injections of 4 mg/kg per day) METH administration. Further, both escalating and binge METH resulted in opposing responses in tissue taken 2 weeks post-METH compared to that taken 48 hours post-METH, including significant decreases in VTA DA content and increases in NAcc DA tissue content. The reported changes in DA tissue content in the NAcc and VTA paralleled findings of tyrosine hydroxylase-positive tissue content as a biphasic response to escalating and binge METH was also observed. However, within the whole striatum, neither escalating METH doses nor binge METH altered levels of DA or tyrosine hydroxylase tissue content. Additionally, DAT immunoreactivity was altered in a biphasic manner in the VTA, but was unchanged in the NAcc, striatum, or substantia nigra, following METH (Keller, et al., 2011). Taken together, these studies in conjunction with the current results reveal the region-specific vulnerability of the VTA to high concentrations of METH.

Toxic Effects of CORT in the VTA

Similar to the METH-induced effects occurring preferentially in the VTA, chronic CORT exposure also resulted in significant increases in PI uptake in the VTA, but not the NAcc or mPFC, in the current set of experiments. Contrary to the present results, Mesquita and colleagues (2007) reported that maternal separation for 14 days during the early post-natal period did not alter DA turnover nor DA or its metabolites, DOPAC and HVA, in the VTA (although a significant increase in DOPAC was found in females

exposed to maternal separation compared to their control counterparts). Additionally, stress via maternal separation did not significantly alter TH-positive or tryptophan hydroxylase-positive immunocytochemistry in either the VTA or the dorsal raphe nuclei (Mesquita, et al., 2007). Haneyuki et al. (1991) reported a slight, but significant, increase in DOPAC levels in the VTA following exposure to a brief (10 min) psychological social stressor and a rise in DOPAC levels in the mPFC following a longer exposure to the social stressor (30 min), suggesting that acute stressors may result in oxidative stress via DA metabolism. These results suggest that CORT-induced activation of DA neurons within the VTA may precede that in terminal areas of projection, such as the mPFC (Kaneyuki, et al., 1991). In concert with these previous reports, the current results suggest that the VTA may be selectively vulnerable to the effects of stress and elevations in CORT levels compared to other regions of the mesocorticolimbic pathway.

CORT, METH, and CORT + METH Failed to Alter Levels of NeuN Immunoreactivity

Unexpectedly and contrary to the hypothesis, the current studies revealed no significant treatment effect on immunoreactivity of the neuronal nuclear protein, NeuN. Although few studies have investigated the effects of acute METH on NeuN immunoreactivity, one recent study found that METH (30 mg/kg) administration in mice resulted in increased apoptosis in approximately 25% of striatal neurons as evidenced by decreased NeuN and increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (Zhu, Xu, & Angulo, 2006). Additionally, vulnerability to METH-induced apoptosis was dependent upon subpopulation as only 21% of DA- and cAMP-regulated projection neurons showed apoptosis compared to 45% of GABA and 29% of cholinergic interneurons (Zhu, et al., 2006). Consistent with the

current results, repeated administration of METH (5 mg/kg for 10 days) during development in adolescent mice resulted in no significant changes in NeuN immunoreactivity within the basal forebrain (Siegel, Park, & Raber, 2011); further, repeated METH exposure did not significantly alter levels of GABAergic or acetylcholinergic neurons in the basal forebrain. Although several methodological differences exist between the aforementioned studies and the current experiment, future studies should investigate the timecourse associated with a loss of NeuN immunoreactivity following a neurotoxic regimen of METH.

CORT and METH Co-exposure: Potential Neuroadaptations in NMDA receptor subunit NR1

The current study revealed no significant changes in NR1 density via western blot analysis following CORT, METH or the combination of CORT and METH. Although no study to date has examined the effects of concurrent exposure to CORT and METH on NR1 subunit density, the present results suggest that NR1 density remains largely unaltered following co-exposure to CORT and METH, although it must be noted that METH exposure resulted in slight decreases in NR1 regardless of pre-treatment. The lack of literature on this topic is surprising given that both CORT and METH individually purportedly result in alterations in the NR1 subunit (see below). However, extensive research suggests that regional differences exist in NMDA receptor expression, including the NR1 subunit (Luo, Bosy, Wang, Yasuda, & Wolfe, 1996). As the current western blot experiments did not allow for detection of regional differences, the possibility that CORT and METH act in concert to significantly alter NR1 subunit density in a region-specific manner cannot be ruled out.

Methamphetamine Exposure: Potential Neuroadaptations in NMDA receptor subunit NR1

Whereas previous studies investigating the effects of METH on NR1 have yielded mixed results, the current study found no significant changes in NR1 density following acute METH exposure. Western blot analysis for the NR1 subunit revealed that subacute exposure to METH (4 mg/kg/day for 14 days) resulted in upregulation in the frontal cortex while both acute (8 mg/kg) and subacute exposure resulted in upregulation of NR1 in the striatum (Kerdsan, et al., 2009). Further, no significant effects of either acute or subacute METH were observed in the hippocampus (Kerdsan, et al., 2009). Simoes and colleagues (2007) also showed upregulation of NR2A expression and no change in NR1 expression when METH was given acutely, and a downregulation of NR1 expression and no change in NR2A expression when METH was given in escalating doses. Protracted withdrawal of 14 days, but not 3 days, from amphetamine administration has also been shown to reduce NR1 subunit density in the VTA, NAcc and PFC, accompanied by a significant decrease in NR1 mRNA only evident in the PFC (W. Lu, Monteggia, & Wolf, 1999). Although not statistically significant, the current study saw a slight decrease in NR1 density in samples containing the VTA, NAcc, and mPFC following 24 hour exposure to METH.

Corticosterone Exposure: Potential Neuroadaptations in NMDA receptor subunit NR1

Similar to the effects of METH on NR1 subunit density, the lack of significant changes in NR1 protein levels following chronic CORT observed in the current set of experiments is in contrast to previous reports, although the literature is mixed in regards

to direction. Cohen et al. (2011) reported a 47% decrease in NR1 protein levels in the postsynaptic density subsynaptic fraction of adult mice following implantation with a CORT pellet for 21 days. In contrast, a persistent upregulation of the NR1 and NR2A subunits in the hippocampus followed early-life maternal separation stress when examined in young (3 months) and aged (18 months) rats (Martisova et al., 2012). In agreement with the current results, Weiland and colleagues (1997) saw no differences in NR1 subunit density in the hippocampus of rats following 10 day subcutaneous implantation with a CORT pellet. Together, the current results along with previous studies, largely involving the hippocampus, demonstrate the regional differences in NR1 subunit density following chronic stress or CORT exposure. Indeed, a recent review paper detailed many differences in glutamatergic transmission, including receptor and release differences (Popoli, Yan, McEwen, & Sanacora, 2012). For example, Yuen and colleagues (2012) showed that surface and total NR1 was decreased up to 70% in the PFC, but not the striatum or hippocampus, following seven days of repeated restraint stress. Further, the effects of chronic restraint stress on NR1 density in the PFC was shown to be dependent upon GR activation as administration of the GR antagonist RU-486 attenuated the observed loss, suggesting that the GR, in part, modulates GLUT receptor expression in the PFC following repeated stress (Yuen, et al., 2012). Given evidence for such regional discrepancies, future experiments should investigate the effects of chronic CORT on individual regions such as the mPFC, NAcc, and VTA. Collectively, these studies demonstrate the complexity of glutamatergic receptor density following both CORT and METH exposure.

Corticosterone and Methamphetamine Co-exposure: Potential Neuroadaptations in NMDA receptor subunit NR2B

Although no study to date has examined the effects of concurrent CORT/stress and METH on NR2B subunit density, the present results suggest that previous CORT has no relation to subsequent METH-induced decreases of NR2B density. This finding is surprising given that both CORT and METH individually produce alterations in the NR2B subunit as well as the NR1 subunit as previously discussed. However, there is strong evidence that regional differences exist, both in toxicity and receptor subunit expression, that may underlie this conundrum. One limitation to the current western blot experiments is that it does not allow for regional differences to be examined. Indeed, select regional vulnerabilities were observed in the current set of studies such that the mPFC and VTA appear to be more susceptible to CORT- and METH-induced toxicity compared to the NAcc. In conjunction with previous reports supporting regional differences in NMDA receptor subunits following stress or CORT exposure (Popoli, et al., 2012), these studies reveal the importance of investigations detailing both the individual and combined effects of CORT and METH in several independent regions of the brain. Together, these studies demonstrate the complexity of glutamatergic receptor density, in particular, the polyamine-sensitive NR2B subunit, following both CORT and METH exposure.

Methamphetamine Exposure: Potential Neuroadaptations in NMDA receptor subunit NR2B

While chronic CORT did not significantly alter the density of the polyamine-sensitive NR2B subunit of the NMDA receptor, METH exposure for 24 hours did result in significant decreases in NR2B density regardless of pre-treatment (DMSO vs. CORT). The decrease in NR2B protein levels observed following METH in current studies are in contrast to the hypothesis and to results previously reported. Davidson and colleagues (2007) found that a neurotoxic regimen of METH did not alter NR2B subunits or their phosphorylation state in the caudate, but did significantly increase levels of PFC NR2B, in addition to increasing AMPA GluR1 subunits and phosphorylated subunits, in adult male rats. Similarly, mice exhibiting conditioned place preference following intermittent METH also had increased expression of NR1, NR2A, and NR2B in the lower midbrain region (Kurokawa et al., 2011). Given the previous reports indicating increased polyamine-sensitive NR2B expression following METH, it is surprising the current study did not achieve similar results. However, significant methodological differences exist between the current study and the aforementioned reports, including METH regimen and regions examined, which may be responsible for the inconsistencies.

Corticosterone Exposure: Potential Neuroadaptations in NMDA receptor subunit NR2B

The current results indicating no significant changes in NR2B subunit density following chronic exposure to CORT are in stark contrast to previous reports of upregulation of the polyamine sensitive subunit in the hippocampus. Weiland and colleagues (1997) showed that subcutaneous implantation with a CORT pellet for 10 days increased levels of mRNA for both the NR2A and NR2B subunits in all regions of the hippocampus examined. Consistent with the results from Weiland et al., Klug and colleagues (2012) showed that chronic treatment with CORT in the drinking water for 3

weeks resulted in a significant increase in NR2B levels within the dorsal hippocampus of mice. In kind with the current NR2B results, another recent study found that NR1 and NR2A, but not NR2B, density within the hippocampus was upregulated following early-life maternal separation stress, an effect which was present in young rats and persisted into late adulthood up to 18 months (Martisova, et al., 2012). Although many of the previous reports detailing the ability of CORT to upregulate NR2B expression and/or function have been conducted in the hippocampal region of the rodent brain, the current results suggest that these effects may be dependent upon the region examined and possibly the basal density of NMDA receptors containing the polyamine sensitive NR2B subunit, which is thought to be high in the hippocampus compared to other limbic structures (Loftis & Janowsky, 2003; Monaghan & Cotman, 1985).

Effects of Combined CORT and METH on HPLC Analysis of Extracellular Dopamine

The current study revealed no significant changes in extracellular DA levels following CORT, METH or the combination of CORT and METH, although a slight, non-significant decrease in DA was observed following exposure to METH for 45 minutes. This is in contrast to the effects associated with dose-dependent increases in extracellular DA collected from co-cultures containing the VTA, NAcc, and mPFC following acute (30 min) exposure to METH, cocaine, or morphine (Nakagawa, et al., 2011). In this study, METH (100 or 1000 μ M; lower concentrations did not significantly evoke a rise in DA) resulted in significant increases in extracellular DA. However, the current study failed to evoke elevations in extracellular DA following acute exposure to a single concentration of METH (100 μ M). Further, Nakagawa and colleagues (2011)

revealed augmentation of extracellular DA following repeated exposure to METH in the triple slice co-culture model. Although the current study did not investigate the effects of repeated METH on subsequent METH-induced changes in extracellular DA content, the present experiments did investigate the possible cross-sensitization between CORT and METH as several *in vivo* studies have demonstrated that prior stress can cross-sensitize the neurochemical and behavioral responses to METH (Piazza, et al., 1990; Piazza & Le Moal, 1997). The current results are not in agreement with previous literature indicating a significant elevation in extracellular DA levels following METH nor reports suggesting CORT can enhance METH-evoked increases in extracellular DA. Although the reasons behind said discrepancies are not yet clear, slight modifications (including age of pups when tissue collection was initiated) in the preparation of rat mesocorticolimbic triple slice co-cultures were undertaken in the current studies, compared to the methods utilized by Nakagawa and colleagues, that may be responsible for the differences.

Effects of METH on HPLC Analysis of Extracellular Dopamine

Regardless of the discrepancies between the current results and previous reports, METH caused a slight, non-significant, decrease in extracellular DA levels compared to vehicle-treated controls, an effect which was not altered by prior exposure to CORT. This slight decrease may be indicative to mild dopaminergic toxicity occurring following exposure to high-concentration METH. In agreement with this, mild, yet significant, toxicity was observed in the VTA area following acute METH in the present studies. Further indications of toxicity were significant decreases in NR2B subunit density following 24 hour METH exposure and slight, non-significant decreases in NR1 subunit density. Since the samples collected for both western blot and extracellular DA content

analysis were collected from whole triple slice co-cultures, it is hard to discern whether dopaminergic toxicity was present in all of the regions or just select regions. In sum, the present set of data indicates METH-induced dopaminergic toxicity in co-cultures.

Effects of CORT on HPLC Analysis of Extracellular Dopamine

Acutely, CORT administration has been shown to increase levels of extracellular DA within the NAcc (Piazza et al., 1996). However, previous reports have also shown that various stressors have differential influences on mesolimbic DA activity, with inescapable cold stress producing decreases in VTA DA neuron activity while restraint stress increased VTA DA neuronal activity (Valenti, Gill, & Grace, 2012). Long-term exposure to stressors, such as that seen with the chronic unpredictable stress paradigm, has been shown to deplete extracellular DA levels within the NAcc (Gambarana et al., 1999). Similar to the results obtained in the current study, Finlay and colleagues (1995) showed that neither acute nor chronic stress altered basal levels of extracellular DA in the PFC, but rather altered levels in response to the benzodiazepine diazepam. Given the range of effects on extracellular DA (within various regions of the brain) associated with stress or increases in CORT levels, the present finding that chronic CORT did not alter basal or METH-induced DA levels is not altogether unexpected. Future experiments should investigate the possibility that CORT has region-specific effects on extracellular DA levels within the VTA, NAcc, and mPFC.

Effects of Combined CORT and METH on HPLC Analysis of Extracellular Glutamate

The present study revealed that chronic pre-exposure to CORT followed by acute exposure to METH for 45 minutes significantly decreased extracellular GLUT levels

compared to vehicle-treated controls. The depressed levels of extracellular GLUT in response to CORT and METH were significantly different from that seen with CORT alone and a trend towards significance ($p=0.10$) compared to vehicle and METH was observed. This is in stark contrast to the *in vivo* effects previously reported by the group of Yamamoto, suggesting that stress and METH act synergistically to enhance striatal GLUT (Matuszewich & Yamamoto, 2004a, 2004b; Quinton & Yamamoto, 2007; Raudensky & Yamamoto, 2007b; Tata, et al., 2007; Tata & Yamamoto, 2008). Although the majority of previous reports have investigated GLUT levels in the hippocampus or striatum following stress and METH, the current results suggest that CORT exposure prior to and during acute METH may result in decreased extracellular GLUT within the mesocorticolimbic pathway.

Perhaps not surprisingly, the combined effects of CORT and METH on extracellular GLUT were reversed by co-exposure to the NMDA receptor antagonist APV during CORT and METH exposure. Notably, the effect of APV on extracellular GLUT was not specific to CORT and METH co-exposure, as APV treatment (regardless of prior exposure) significantly increased GLUT levels. Although no significant effects of CORT, METH, or APV on extracellular DA levels were found, the slight decrease in extracellular DA observed following METH exposure was also reversed by co-exposure to APV. Indeed, several studies have noted the interactions between GLUT and DA within areas of the mesocorticolimbic reward pathway. In particular, Del Arco and Mora (Del Arco & Mora, 2001) showed that stimulation of prefrontal glutamatergic NMDA receptors dose-dependently reduced basal and stimulated DA release in the PFC. Further investigations have revealed an opposite effect on DA levels within the NAcc such that

blockade of NMDA receptors increased basal levels of DA and DA metabolites (Del Arco, et al., 2008). Taken together, these studies suggest that GLUT receptors, and the NMDA receptor in particular, may be modulating extracellular levels of DA and GLUT within areas of the mesocorticolimbic pathway.

Effects of METH on HPLC Analysis of Extracellular Glutamate

Though many *in vivo* studies have found increases in extracellular GLUT within the rodent striatum following high doses of METH (Abekawa, et al., 1994; Bustamante et al., 2002; Mark, et al., 2004; Nash & Yamamoto, 1992; Stephans & Yamamoto, 1994), the finding that METH decreased GLUT levels, although opposite in nature to the hypothesis, is not without previous precedence. Previous reports have suggested both a time- and region-dependent effect of acute METH on extracellular GLUT levels, such that, following an acute administration of METH (1 mg/kg), GLUT levels decreased in the VTA, substantia nigra compacta, and caudate-putamen while GLUT levels rose in the NAcc (Zhang, Loonam, Noailles, & Angulo, 2001). Following seven days of METH (1 mg/kg) administration, these effects were reversed to above baseline levels in the VTA, substantia nigra compacta, and caudate-putamen whereas the NAcc showed an increase of much less magnitude than following acute administration (Zhang, et al., 2001). Further, Pereira and colleagues (2012) showed that GLUT, GABA, and glutamine striatal tissue contents were significantly decreased 72 hours after a single administration of METH (30 mg/kg). As many studies have shown the neurotoxic effects associated with METH, possibly resulting from excitotoxicity, it is of the utmost importance to determine the regional and time-dependent effects of both acute and chronic METH on extracellular GLUT.

Effects of CORT on HPLC Analysis of Extracellular Glutamate

Surprisingly, the current study found no effect of chronic CORT on extracellular GLUT. Many investigations have noted enhanced extracellular GLUT in limbic and cortical areas, such as the PFC and NAcc, following exposure to acute stressors or elevation in CORT levels (Lowy, Wittenberg, & Yamamoto, 1995; Moghaddam, 1993; Reznikov et al., 2007; Venero & Borrell, 1999). However, the effects of chronic stress or chronic CORT administration on extracellular GLUT levels are less well defined. Repeated tail-pinch stressors had no effect on extracellular GLUT levels within the hippocampus, while each subsequent stressor decreased extracellular GLUT levels within the PFC (Bagley & Moghaddam, 1997). Thus, chronic stress may differentially alter levels of GLUT within the PFC compared to the hippocampus. Future studies should investigate the effects of repeated exposure to stressors or CORT on extracellular GLUT within the PFC compared to mesolimbic structures, including the NAcc and VTA.

Implications and Future Directions

Many studies have revealed parallel neuroadaptations following chronic stress or exposure to stimulants, such as METH. Indeed, several human studies have shown that METH administration leads to persistent alterations in the stress hormone cortisol or HPA axis dysfunction (King, Alicata, Cloak, & Chang, 2010; Li et al., 2013). Preclinical studies with non-human primates as well as rodents have shown that CORT levels remain elevated up to 24 hours following either acute or chronic METH administration (Grace et al., 2008; Madden et al., 2005). Further, rodent models of amphetamine administration have shown that CORT levels, both basal and amphetamine-induced, are altered by

environmental stressors such as differential rearing conditions (Stairs, et al., 2011). The present results suggest that the NMDA receptor may be implicated in CORT and METH-induced toxicity within the mPFC, an area of the brain crucial to executive function and inhibitory control. However, it is also important to note the significance of phosphorylation state when investigating the NMDA receptor, as tyrosine phosphorylation of the NR2B, but not the NR2A, subunit has been implicated in synaptic plasticity of the rat striatum (Lau & Huganir, 1995; Menegoz, Lau, Herve, Huganir, & Girault, 1995). Future studies should investigate the possibility of increased expression of the polyamine sensitive NR2B subunit and the potential role of changes in phosphorylation state of the NMDA receptor in mediating the effects of chronic CORT within areas of the mesocorticolimbic pathway. The current studies suggest that glutamatergic signaling and NMDA receptors may play a significant role in stimulant-associated brain damage and cognitive disinhibition often associated with substance abuse. Further, the present results suggest that antagonism of glucocorticoid or NMDA receptors may be valuable therapeutic targets in treating individuals with co-morbid METH dependence and HPA axis alterations.

In conclusion, initial studies demonstrated that long-term exposure to a high, yet physiologically relevant, concentration of CORT can potentiate NMDA receptor-mediated neurotoxicity within the VTA, but not the NAcc, in a GR-dependent manner. In agreement with previous data regarding hippocampal toxicity, these results suggest that chronic CORT may lead to an increase in the expression and/or function of NMDA receptor systems in an area of the brain that is critical for drug reward and reinforcement. A second set of experiments demonstrated that chronic CORT can sensitize the mPFC,

but not the NAcc or VTA, to subsequent METH-induced toxicity in a NMDA receptor-dependent manner. Co-exposure to CORT and METH also resulted in a significant decrease in extracellular GLUT, an effect which was reversed by NMDA receptor antagonism. Together, these studies suggest that long-term exposure to CORT enhances glutamatergic signaling in response to future excitotoxic events, such as METH exposure, in areas of the brain critical for reward.

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PROFESSIONAL PUBLICATIONS

Journal Articles

- Butler TR, **Berry JN**, Sharrett-Field LJ, & Prendergast MA (2013; in press). Long-term ethanol and corticosterone co-exposure sensitize the hippocampus to insult during ethanol withdrawal in an NMDA GLUN2B subunit-dependent manner. *Alcoholism: Clinical and Experimental Research*
- Sharrett-Field LJ, Butler TR, Reynolds AR, **Berry JN**, & Prendergast MA (2013; in press). Sex differences in neuroadaptation to alcohol and withdrawal toxicity. *Pflügers Archiv - European Journal of Physiology*. 465(5): 643-654
- Sharrett-Field LJ, Butler TR, **Berry JN**, Reynolds AR, & Prendergast MA (2013). Mifepristone pre-treatment reduces ethanol withdrawal severity *in vivo*. *Alcoholism: Clinical and Experimental Research*. 37(8): 1417-1423.

- Berry JN**, Sharrett-Field LJ, Butler TR, & Prendergast MA (2012). Temporal dependence of cysteine protease activation following excitotoxic hippocampal injury. *Neuroscience*. 222: 147-158. [PMCID: PMC3433750].
- Berry JN**, Neugebauer NM, & Bardo MT (2012). Reinstatement of methamphetamine conditioned place preference in nicotine-sensitized rats. *Behavioural Brain Research*. 235(2): 158-165. [PMCID: PMC3445737].
- Butler TR, Self RL, Mulholland PJ, Smith KJ, Sharrett-Field LJ, **Berry JN**, Pauly JR, & Prendergast MA (2010). Selective vulnerability of hippocampal cornu ammonis 1 pyramidal cells to excitotoxic insult is associated with the expression of polyamine-sensitive N-methyl-D-aspartate-type glutamate receptors. *Neuroscience*. 165(2): 525-534. [PMCID: PMC2794945]
- Butler TR, Smith KJ, **Berry JN**, Sharrett-Field LJ, & Prendergast MA. (2009). Sex differences in caffeine neurotoxicity are exacerbated by prior ethanol exposure and withdrawal. *Alcohol and Alcoholism*. 44(6): 567-574. [PMCID: PMC2842109]

Manuscripts Submitted/in Preparation

- Berry JN**, Sharrett-Field LJ, Reynolds AR, & Prendergast MA (In preparation for *Brain Research*). Corticosterone enhances N-methyl-D-aspartate receptor signaling to promote ventral tegmental area neurotoxicity in the reconstituted mesolimbic dopamine pathway.
- Berry JN**, Butler TR, Sharrett-Field LJ, Reynolds AR, & Prendergast MA (In preparation for *Alcoholism: Clinical and Experimental Research*). Exposure to Corticosterone During Ethanol Exposure and Withdrawal Augments the Loss of Synaptophysin Immunoreactivity in a NR2B-dependent Manner.
- Reynolds AR, **Berry JN**, Sharrett-Field LJ, & Prendergast MA. (Submitted to *Journal of Neurochemistry*). Ethanol withdrawal is required to produce loss of mature hippocampal neurons after chronic intermittent ethanol exposure *in vitro*.

Professional Presentations

- Berry JN**, Sharrett-Field LJ, Reynolds AR, & Prendergast MA. 2013. Co-exposure to corticosterone and ethanol augments the loss of synaptophysin in a NR2B-dependent manner. Abstract submitted for poster presentation, *Research Society on Alcoholism*, Orlando, FL.
- Reynolds AR, **Berry JN**, Sharrett-Field LJ, & Prendergast MA. 2013. Chronic intermittent ethanol exposure produces loss of mature neurons in hippocampal

slice cultures. Abstract submitted for poster presentation, *Research Society on Alcoholism*, Orlando, FL.

Sharrett-Field LJ, **Berry JN**, Reynolds AR, & Prendergast MA. 2013. Startle reactivity predicts and is altered by intermittent access to ethanol in the outbred long evans rat. Abstract submitted for poster presentation, *Research Society on Alcoholism*, Orlando, FL.

Berry JN, Sharrett-Field LJ, Reynolds AR, & Prendergast MA. 2013. Exposure to corticosterone during ethanol exposure and withdrawal augments the loss of synaptophysin immunoreactivity in a NR2B-dependent manner. Abstract for poster presentation, Bluegrass Chapter Society for Neuroscience Spring Neuroscience Day, Lexington, KY.

Berry JN, Sharrett-Field LJ, Reynolds AR, & Prendergast MA. 2012. Chronic corticosterone sensitizes the mesolimbic dopamine reward pathway to excitatory NMDA receptor activity. Abstract for poster presentation, *Society for Neuroscience*, New Orleans, LA.

Sharrett-Field LJ, Butler TR, **Berry JN**, Reynolds, AR, Prendergast MA. 2012. Short term ethanol exposure induces calpain dependent α -spectrin proteolysis and neurodegeneration in the hippocampus. Abstract for poster presentation, Research Society on Alcoholism, San Francisco, CA.

Berry JN, Sharrett-Field LJ, Butler TR, Reynolds AR, & Prendergast MA. 2011. Time-dependence of cysteine protease activation following excitotoxic hippocampal injury. Abstract for poster presentation, Society for Neuroscience, Washington, D.C.

Sharrett-Field LJ, Butler TR, **Berry JN**, & Prendergast MA. 2011. Glucocorticoid antagonist diminishes alcohol withdrawal severity in rats following EtOH exposure using a novel binge paradigm. Abstract for poster presentation, Research Society on Alcoholism, Atlanta, GA.

Snyder KN, **Berry JN**, & Prendergast MA. 2011. Development of an organotypic model to study the mesolimbic dopamine reward pathway. Abstract for poster presentation. Society for the Promotion of Undergraduate Research. Lexington, KY.

Berry JN, Sharrett-Field LJ, Butler TR, & Prendergast MA. 2010. Time-dependence of distal-to-proximal hippocampal neurodegeneration produced by *N*-methyl-D-aspartate receptor activation. Abstract for poster presentation, Society for

Neuroscience, San Diego, CA; Bluegrass Chapter of SfN Spring Neuroscience Day, Lexington, KY.

Butler TR, Smith KJ, Sharrett-Field LJ, **Berry JN**, & Prendergast MA. 2009. Hippocampal CA1 region vulnerability to neurotoxic insult: involvement of polyamine-sensitive NMDA receptors. Abstract for poster presentation, Society for Neuroscience, Chicago, IL.

Berry JN, Neugebauer NM, & Bardo MT. 2007. Reinstatement of extinguished methamphetamine conditioned place preference. Abstract for poster presentation, Kentucky Psychological Association, Lexington, KY; abstract for oral presentation, Midwestern Psychological Association, Chicago, IL.

HONORS & AWARDS

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| 2013 | Behavioral Neuroscience and Psychopharmacology Graduate Student Achievement Award, University of Kentucky |
| 2013 | RSA Conference Student Merit Award, Research Society on Alcoholism |
| 2010 | Bluegrass Chapter of Society for Neuroscience Spring Neuroscience Day Graduate Student Poster Award, University of Kentucky |
| 2007 | Departmental Honors in Biology and Psychology, University of Kentucky |
| 2006 | Mary Agnes Gordon Undergraduate Award, University of Kentucky, Department of Psychology |
| 2003 – 2007 | University Honors Program, University of Kentucky |