

**VENTRAL HIPPOCAMPAL CORTICOSTERONE MEDIATES ACCUMBAL SHELL  
DOPAMINE OUTPUT: POTENTIAL LINK BETWEEN STRESS AND INCENTIVE  
SALIENCE IN CONTROL CONDITIONS AND IN PROTRACTED AMPHETAMINE  
WITHDRAWAL**

By  
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Division of Basic Biomedical Sciences

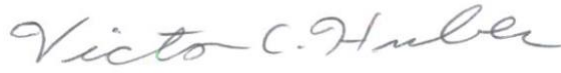
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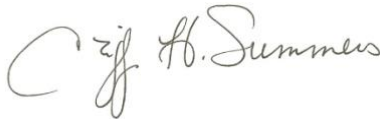
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## **Dedication**

In this past 6-year journey I have met many points in which I could not progress further on my own, or thought that I could not, and would like to thank those who helped me continue to move forward. First and foremost, I owe great thanks to my mentor Dr. Gina Forster. In addition to providing me with technical and theoretical research training, Dr. Forster provided me with numerous opportunities to advance my research and career goals, while encouraging me to think and act independently with the perfect amount of support. I would also like to thank those who provided me with welcome mats, stepping stools, footholds, and crash pads into the world of neuroscience: Shelly Dickinson, PhD, Kevin Crisp, PhD, and Jay Demas, PhD at St. Olaf College; Lee Baugh, PhD, Brian Burrell, PhD, Victor Huber, PhD, Robin Miskimins, PhD, Ken Renner, PhD, Samuel Sathyanesan, PhD, Cliff Summers, PhD and Michael Watt, PhD at the University of South Dakota (to name a few). You have all provided me with unimaginable guidance and support through classroom and laboratory teaching experiences, learning opportunities in seminars and workshops, time and help in reviewing manuscripts and grant proposals, and through personal insight provided off the clock. THANK YOU!

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“So remember to look up at the stars and not at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at. It matters that you don’t just give up. Unleash your imagination. Shape the future.”

- Stephen Hawking, Brief Answers to the Big Question

## Abstract

Stress can increase the reward neurotransmitter dopamine in the nucleus accumbens shell, where dopamine is associated with incentive salience. However, stress can also reduce accumbal shell dopamine release during psychostimulant withdrawal. The resulting dysphoria can directly drive drug behaviors and predict relapse. Stress and psychostimulant withdrawal also induce neurobiological changes in the ventral hippocampus, a brain region associated with drug reward and stress responses. Systemically, stress induces peripheral release of the glucocorticoid stress hormone corticosterone (cortisol in humans). Corticosterone can modulate local excitation in the ventral hippocampus through its glucocorticoid and mineralocorticoid receptors. Ventral hippocampal excitation can enhance accumbal dopamine release in control conditions, and reinstate drug behaviors during amphetamine withdrawal. Therefore, we used a validated rat model of amphetamine pretreatment and withdrawal to test whether corticosterone in the ventral hippocampus can directly alter accumbal dopamine release in control conditions and in protracted amphetamine withdrawal. Repeated amphetamine exposure *enhanced* the ability of acute restraint stress to increase free extracellular corticosterone levels in the ventral hippocampus during protracted withdrawal. This effect was not mediated by alterations to peripheral corticosterone stress responses or local corticosteroidogenic enzymes. Instead, the effect may be mediated by alterations in central corticosterone binding globulin, which cannot be assessed by microdialysis. Replicating the ventral hippocampal corticosterone stress response by infusing stress-relevant concentrations of corticosterone into the ventral hippocampus rapidly *enhanced* accumbal dopamine output in control conditions but produced a biphasic *reduction* in amphetamine withdrawal. Selectively blocking activation of corticosterone's glucocorticoid-, mineralocorticoid-, or cytosolic receptors in the ventral hippocampus blocked the effects in control- and withdrawal conditions. Therefore, we conclude corticosterone's ability to rapidly alter accumbal dopamine output requires concomitant activation of *both* excitatory membrane mineralocorticoid receptors *and* disinhibitory cytosolic glucocorticoid receptors. Our findings suggest the differential effects of ventral hippocampal corticosterone on accumbal dopamine release may help enable stress to direct goal-oriented behavior in control conditions and produce the dysphoric states that drive relapse in psychostimulant withdrawal. These findings emphasize a *bona fide* role for the ventral hippocampal corticosterone system in contributing to positive reinforcement of initial drug use and dysphoric states that negatively reinforce drug dependence.



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Advisor's Approval Signature

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**Preface: The hippocampus as a neural link between negative affect and vulnerability for psychostimulant relapse.**

**Abstract**

Psychostimulant dependence (including cocaine, amphetamine, and methamphetamine) is a chronic relapsing disorder with significant personal, health, and financial burdens. Attempts at abstinence produce a severe and protracted withdrawal syndrome characterized by stress hypersensitivity that can facilitate drug craving, anxiety, and dysphoria. These negative withdrawal symptoms can induce relapse, maintaining the addiction cycle. The hippocampus mediates cognitive, emotional, and endocrine responses to stressors. The ventral hippocampus is in a pivotal position to regulate the mesoaccumbal dopamine reward system. It also interacts with serotonergic and glucocorticoid systems that mediate anxiety and stress responsiveness. Psychostimulant actions on the hippocampus induce long-term changes to these systems and impact the process of adult neurogenesis in the hippocampus, which may facilitate drug dependence by altering drug-cue learning and emotional regulation. Multiple studies indicate that psychostimulant-induced hippocampal neuroadaptations heighten hippocampal-mesoaccumbal activity to amplify drug- and drug-cue responses while persistent dysregulation of hippocampal emotional systems potentiate negative affect. Understanding how psychostimulants modulate the hippocampus to alter hippocampal-mesoaccumbal activity – and how hippocampal neurogenesis influences drug-related memories and reward – is important for identifying novel treatment

strategies that can ameliorate negative affect and relapse vulnerability in psychostimulant addiction.

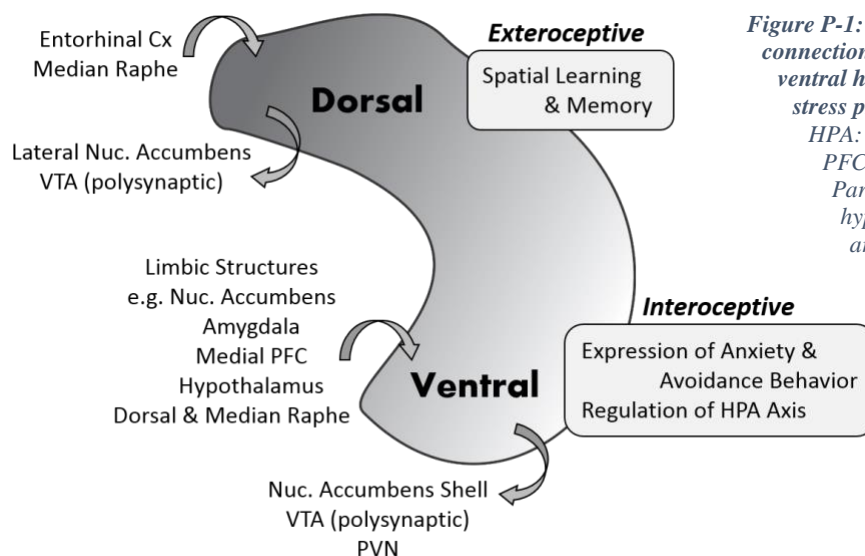
## **1. Introduction**

**1.1 The problem of stimulant abuse.** Abuse of psychostimulants such as cocaine and amphetamines affects millions of people worldwide, as psychostimulants are the second most widely abused class of illicit drug globally behind marijuana [1-5]. In general, drug addiction and subsequent relapse vulnerability are thought to occur through counter-adaptive neurochemical changes within brain circuits that normally conserve an emotional homeostasis [6-8].

Dysregulation of the homeostatic system – through genetics, environment (stress), history of drug taking, or current emotive states – produces susceptibility to become dependent and to relapse during long term abstinence [9, 10]. Psychostimulants produce a severe and protracted withdrawal syndrome which includes symptoms of stress hypersensitivity, intense drug craving, anxiety, and dysphoria [11-16]. These symptoms are reproduced in animal models [17-21], and can induce craving and relapse in humans [13, 22, 23], thus maintaining the addiction cycle [24-27]. The underlying mechanisms that enable stress-sensitive and dysphoric states in withdrawal to induce relapse are thought to involve alterations to the mesolimbic dopamine reward system and anti-reward/stress systems [9, 26, 28] that include the hippocampus [28-30]. Currently, no medications have proven effective for treating psychostimulant withdrawal [13, 16, 31]. Thus, understanding the neurobiology underlying the aversive states during psychostimulant withdrawal is an essential component of relapse prevention [32].

**1.2. The hippocampus, stress and addiction.** The hippocampus, a brain region associated with

spatial learning and memory, has been established as a critical region for reward- and stress-associated responses and drug-seeking behaviors [30, 33-37]. Exposure to conditioned contextual cues and aversive or stressful stimuli are powerful triggers of drug cravings [38-41] and are associated with activation of limbic brain regions, including the hippocampus, in both human and rodent models [42-46]. Dorsal and ventral subdivisions of the rodent hippocampus have been proposed based on anatomical connectivity and behavioral output [47-51]. The rodent dorsal hippocampus, analogous to the human posterior hippocampus, receives *exteroceptive* information from the entorhinal cortex and has a major role in rapid spatial learning [52] (Figure 1). The ventral hippocampus, analogous to the human anterior hippocampus, receives *interoceptive* information through reciprocal connections to limbic regions that modulate motivational and affective states; the other limbic brain regions involved include the nucleus accumbens, amygdala, medial prefrontal cortex, and hypothalamus [50-54] (Fig. P-1). Notably, both regions of the hippocampus are involved in memory formation [55]; dorsal neurons form contextual representations of specific single events while ventral neurons form representations of multiple events (related by a distinct context) over time [56].



**Figure P-1: Schematic of afferent/efferent connections and functions of the dorsal and ventral hippocampus related to reward and stress processes.** Abbreviations: Cx: Cortex; HPA: Hypothalamic-pituitary-adrenal; PFC: Prefrontal cortex; PVN: Paraventricular nucleus of the hypothalamus; VTA: Ventral tegmental area.

The subiculum, the major output structure of the hippocampus, provides projections to the nucleus accumbens, which also receives input from ventral tegmental area (VTA) dopamine terminals [34, 57-59]. The nucleus accumbens integrates affective and motivational information to produce goal-directed behavioral output [60-62]. Thus, the hippocampus is poised to play an important role in mediating the effects of drugs of abuse (e.g. psychostimulants) through its interactions with the mesoaccumbal dopamine system. Importantly, the dorsal and ventral hippocampus may differentially regulate accumbal activity [60, 63], since the ventral subiculum projects to the medial shell of the nucleus accumbens while the dorsal subiculum projects to the more lateral accumbens core (Fig. P-1) [51, 54, 64]. The dorsal and ventral hippocampus also influence accumbal activity indirectly, via multi-synaptic projections to the VTA [65-67] (Figure 1). Consequently, glutamatergic output from the hippocampus facilitates dopaminergic activity in the mesolimbic dopamine pathway [34, 57, 68-69]. In the nucleus accumbens shell, this communication is vital for forming place-reward associations [70-72] and mediating reward salience [63]. Thus, context-related processing within the hippocampus may drive reward-related processes mediated by the nucleus accumbens.

The hippocampus also regulates anxiety and avoidance behaviors. Anxiety is an innate response coordinated to protect an animal from potential harm, which is linked to maximizing chances of reward in approach-avoidance conflict situations. The hippocampus has been proposed to underlie anxiety behaviors by detecting novelty or uncertainty [73, 74] and then increasing attention and behavioral inhibition [75, 76]. However, maladaptive changes to the circuits underlying this response can constrain normal functioning and lead to a disruptive pathological state.

The *ventral* hippocampus plays a particularly predominant role in mediating



anxiety/avoidance behaviors. For example, glutamatergic activation of the ventral hippocampus is important for expressing anxiety-like behaviors [77, 78] and lesioning the ventral – but not dorsal – hippocampus reduces innate avoidance behavior in unconditioned anxiety tests, and reduces conditioned responding to anxiogenic cues [79-84]. Moreover, a recent study in humans found that the anterior (ventral) hippocampus is necessary for passive avoidance behavior [85], and studies in rats and humans have shown that increased activity between the ventral/anterior hippocampus and the medial prefrontal cortex is necessary for expressing anxiety in anxiogenic environments [86-89]. Also, activating basolateral amygdala (BLA) inputs to the ventral hippocampus increases – while inhibition decreases – anxiety-like behaviors [90]. Together, these findings suggest that activation of the ventral hippocampus by glutamatergic input from the BLA and its subsequent communication with regions like the prefrontal cortex is essential for the appropriate expression of anxiety/avoidance behaviors.

Related to its involvement in emotional regulation, the ventral hippocampus also exerts influence on the hypothalamic-pituitary-adrenal (HPA) axis and coordinates stress responses [36, 91, 92] (Figure 1). The HPA axis organizes neuroendocrine responses to physical and psychogenic stressors through release of the glucocorticoid hormone cortisol (humans) or corticosterone (rodents) [92]. The hippocampus is the primary target for glucocorticoids in the brain [93] and the ventral subiculum is thought to be the primary limbic region that utilizes glucocorticoid feedback to inhibit HPA axis activity [91, 94-96]. This feedback inhibition is mediated through corticosteroid activation of corticosterone's mineralocorticoid (MR) and glucocorticoid (GR) receptors that are both cytosolic (genomic) and membrane-bound (non-genomic) [96-99].

Cytosolic MRs (cMRs), with restricted expression (highest in the hippocampus), have ten-fold higher affinity for corticosterone than GRs, and are ~90% occupied under basal conditions [100-103]. They are attributed with regulating HPA inhibition at basal corticosterone levels, and thus determine HPA “set point” [96, 104-108]. cMRs also sustain cellular stability, which maintains stress sensitivity thresholds and preserves limbic network communication [97, 103, 107, 109, 110]. Cytosolic GRs (cGRs) are ubiquitously expressed, with high expression in the hippocampus [95], and regulate delayed feedback inhibition of HPA activity after diurnal corticosterone peaks and acute stress [92, 96, 104-105]. cGRs are also attributed with normalizing neuronal excitability in response to stress and normalizing network activity, which dampens initial stress responses, and promotes adaptive stress coping [107, 109, 110].

Corticosterone stress responses that occur too quickly to attribute to genomic effects are credited to activation of non-genomic receptors in the hippocampus (and other regions) that are often membrane-bound (mMRs/mGRs). Membrane receptors typically have  $\geq 10$ -fold lower affinity for corticosterone than their cytosolic counterparts [97, 103, 108] and thus act as hippocampal “cortico-sensors” [99, 111]. mMRs rapidly and reversibly enhance excitatory glutamatergic transmission in the hippocampus [97, 99, 107]; they contribute to rapid inhibition of HPA activity and activate rapid and reversible behavioral stress responses important for appraisal and coping [99, 110]. mGRs have lower corticosterone affinity than mMRs and augment inhibitory GABAergic interneuronal transmission [112] to suppress excitability; they also promote spinogenesis [97, 113]. Alterations in these receptors’ expression, function, and ratios relative to one another – especially within the hippocampus – can diminish stress responsiveness and coping ability, which is associated with multiple disease states, including depression and psychostimulant withdrawal [113, 114].

Glucocorticoid stress responses in the hippocampus also vary based on hippocampal region (dorsal vs. ventral): acute foot shock rapidly increases corticosterone levels in the dorsal hippocampus, followed by a more delayed elevation in the ventral hippocampus [115]. Also, acute swim stress *decreases* long-term potentiation (LTP) in the *dorsal* hippocampus, but *increases* LTP in the *ventral* hippocampus [116]. This differential response may temporarily suppress the dorsal hippocampus' cognitive cortical communication and facilitate ventral hippocampal transmission of emotional information [117].

**1.3 Goals of this review.** Overall, the ventral hippocampus is in a pivotal position to play a key role in addictive processes via its role in modulating activity of reward and stress pathways such as the mesoaccumbal dopamine system and HPA axis respectively. This review will provide evidence for psychostimulant-induced changes in the hippocampus leading to negative affect that promotes psychological withdrawal symptoms and maintains the cycle of psychostimulant dependence. Specifically, this review will evaluate and integrate various studies concerning alterations of hippocampal activity and structural plasticity due to chronic drug exposure that contribute to the pathophysiology of drug abuse through maladaptive reward responses and/or the promotion of dysphoric states. In doing so, potential mechanisms underlying psychostimulant withdrawal symptoms and relapse to drug-seeking will be revealed and future directions identified.

## **2. Psychostimulants and hippocampal-mesoaccumbens circuitry**

The mesoaccumbal dopaminergic system (VTA to nucleus accumbens) is involved in reinforcement learning and motivated behavior. Dopamine release in the nucleus accumbens

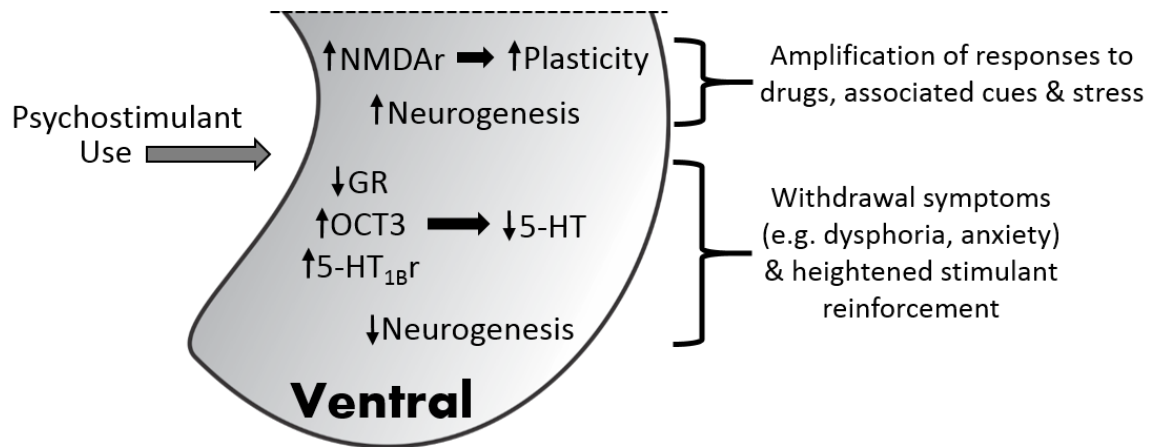
shell is associated with reward salience [63] and drug/reward context conditioning [118], and is enhanced by drug use [42, 118], drug-predictive contexts [118, 119], and during novel environment exploration [120]. In line with its role as a novelty detector, the ventral hippocampus controls the novelty-induced dopamine response in the nucleus accumbens [73]. Novelty-induced activation of the ventral hippocampal-nucleus accumbens pathway is thought to be important for long term memory formation [121]. In support of this, place-reward associations depend on communication between the ventral hippocampus and the nucleus accumbens shell [68, 69]. Likewise, neuronal activity between the nucleus accumbens, hippocampus, and prefrontal cortex during goal-directed behavior learning is believed to contribute to reward-context memory consolidation and strengthening [122-125]. Finally, co-activation of the anterior (ventral) hippocampus and VTA dopamine neurons is linked to long-term reward-related memory enhancement [126, 127]. Thus, reward enhances memory formation, and this effect is closely linked to reward-context engagement of the hippocampal-mesoaccumbal pathway.

The dopamine system has long been associated with stress/aversion as well as reward-related behaviors [128, 129]. For example, stress increases dopamine levels in the nucleus accumbens shell (but not core) [130]. Preliminary studies in rats suggest that mimicking the hippocampal glucocorticoid stress response [132-134] by infusing corticosterone into the ventral subiculum stimulates dopamine efflux in the nucleus accumbens shell [29], thus indicating a role for the ventral hippocampus in enabling stress to enhance accumbal dopamine output. Stressors also increase VTA dopamine activity, and this increase is dependent upon ventral hippocampal activity [135]. The ventral hippocampus-VTA dopamine pathway is also potentiated in mice with increased social avoidance after chronic social defeat stress, and is necessary for this behavioral outcome [136]. Thus, it is suggested that the ventral hippocampus uses prior

experience to bias the responsive state of accumbal dopamine [135]. In line with this suggestion, mice with increased avoidance behavior following chronic stress also display increased VTA dopamine neuron burst firing [137, 138]. Therefore, a behaviorally salient stimulus (aversive or rewarding) within a given context would heighten activation of the ventral hippocampus-accumbens pathway.

The ventral hippocampal-nucleus accumbens pathway also influences psychostimulant responses. Rats with greater dopaminergic responses to novelty will self-administer psychostimulants more readily [139, 140] and rats with repeated cocaine exposure display enhanced accumbal dopamine responses to glutamatergic stimulation of the ventral hippocampus [141]. This is likely reflective of the finding that repeated cocaine exposure and withdrawal selectively potentiates ventral hippocampal input to the nucleus accumbens shell [142, 143]. Furthermore, rats that exhibit behavioral sensitization to amphetamine display enhanced VTA neuronal firing and accumbal dopamine output, and these behavioral and neurophysiological effects are dependent on ventral hippocampal input [144, 145]. Hippocampal activity is also associated with psychostimulant-induced conditioned place preference (CPP) acquisition and expression [146, 147]. For example, lesions or inactivation of the hippocampus inhibit CPP acquisition and context-induced drug-seeking behavior [148-152]. Specifically, interactions between ventral hippocampal glutamatergic projections to neurons expressing postsynaptic D1 dopamine receptors in the nucleus accumbens shell contribute to drug-context memory formation and subsequent drug-seeking reinstatement [37, 153, 154]. Thus, ventral hippocampal facilitation of accumbal dopamine may generate drug-seeking behavior. Further, ventral hippocampal inhibition reduces cocaine- cue- or context-induced reinstatement of drug-seeking behavior [148, 149, 155, 156] and its activity primes context-dependent relapse to drug-seeking for cocaine or

d-amphetamine [37, 154, 158]. Overall, it appears that ventral hippocampal enhancement of accumbal dopamine activity likely promotes storage and retrieval of drug reward information that underlies drug-seeking behaviors (Fig. P-2).



*Figure P-2: Overview of the effects of psychostimulant use on the ventral hippocampus that lead to increased sensitivity to psychostimulants, cues, stress and withdrawal symptoms. As discussed in the text, repeated psychostimulant exposure may either increase or decrease neurogenesis in the hippocampus under differing conditions, with either outcome contributing to the symptoms of dependence. Abbreviations: 5-HT: Serotonin; GR: Glucocorticoid receptor; OCT3: Organic cation transporter 3.*

The mechanisms by which psychostimulants enhance ventral-hippocampal-regulated dopamine activity are not fully understood. Stress and repeated cocaine exposure independently increase LTP in the ventral hippocampus [116, 159]. Interestingly, acute *stress-induced* hippocampal plasticity is mediated by MRs and GRs in the ventral hippocampus; whereas *cocaine-induced* hippocampal plasticity seems to instead involve D2 dopamine receptors [116, 159, 160]. Related, repeated cocaine increases trafficking of glutamate receptors toward the membrane in the rat hippocampus [161], suggesting that psychostimulant-induced changes in hippocampal glutamate receptor availability contribute to increased hippocampal excitability and enhanced elevation of accumbal dopamine [141]. Repeated amphetamine exposure also results in a reduced GR to MR ratio in the ventral hippocampus [114], which could further alter hippocampal excitability [97, 108] and hippocampal-accumbens activity. Further, repeated

psychostimulant exposure alters neurotransmitter and endogenous neuropeptide levels in the hippocampus. For example, intrahippocampal oxytocin is decreased following chronic cocaine whereas, exogenous administration inhibits psychostimulant-induced behaviors [162]. Oxytocin alters hippocampal excitability by increasing the firing rate of inhibitory interneurons, likely influencing hippocampal terminal regions including the mesoaccumbal dopaminergic system [163]. Together, these findings suggest that psychostimulants can alter synaptic plasticity in the ventral hippocampus, facilitating hippocampal-accumbal pathways to amplify responses to drug reward- or stressor- associated cues (Figure 2).

### **3. Psychostimulants and hippocampal affect regulation: spotlight on serotonin and glucocorticoids**

A critical modulator of hippocampal activity is serotonin (5-HT). The serotonergic median raphe nucleus innervates the entire dorsal-ventral axis of the hippocampus while the ventral hippocampus receives additional projections from the dorsal raphe nucleus [162, 163] (Figure 1). Thus, the ventral hippocampus receives a higher density of serotonergic innervations than the dorsal hippocampus [164]. The expression of 5-HT receptors is also differentiated along the dorsal-ventral axis of the hippocampus [165], which supports distinct 5-HT contributions to regionally distinct hippocampal functions.

Various stressors increase 5-HT levels in the hippocampus [166-173], and this is thought to be mediated by GR activation [114, 173, 174]. In rats, total brain 5-HT depletion increases stress sensitivity and abolishes stress adaptation [175], and specific 5-HT depletion in the ventral hippocampus increases anxiety-like behavior [176]. This supports the role of the ventral hippocampus as regulating anxiety behavior, and comports findings that suggest 5-HT acts as an

inhibitory modulator in the hippocampus by activating inhibitory 5-HT<sub>1A</sub> receptors [177-183]. For example, 5-HT<sub>1A</sub> receptor activation in the hippocampal dentate gyrus inhibits LTP and impairs fear-related memory acquisition and consolidation [184-186]. Also, post-stress injection of a selective 5-HT reuptake inhibitor or activation of 5-HT<sub>1A</sub> receptors in the hippocampus prevent stress-induced behavioral deficits [187-189]. Overall, increased 5-HT in the hippocampus seems to be important for repeated stress habituation, while reduced ventral hippocampal 5-HT heightens anxiety [173, 176, 190, 191].

A reciprocal and regulatory interaction exists between the serotonergic and glucocorticoid systems [192-194]. Systemic corticosterone enhances – and blocking corticosterone synthesis or GRs reduces – hippocampal 5-HT turnover and release [114, 195, 196]. These and other findings suggest that hippocampal GR activation in response to stress enhances hippocampal 5-HT transmission [114, 175], which may hold implications for behavioral and emotive stress responses such as anxiety [173, 176]. For example, many antidepressants that decrease anxiety states increase GR expression and 5-HT transmission [197]. In relation to psychostimulant use, chronic amphetamine pretreatment reduces GR protein expression in the ventral hippocampus and abolishes the 5-HT response to physiologically relevant hippocampal corticosterone levels after 24 hours of withdrawal [114], when heightened anxiety states emerge [198]. Overall, blunted stress-induced 5-HT signaling in the ventral hippocampus may contribute to negative affect during psychostimulant withdrawal.

Interestingly, rats with high anxiety behavior and diminished stress-induced 5-HT release also have increased levels of 5-HT transporter (SERT) in the raphe and hippocampus, suggesting enhanced 5-HT clearance from the synaptic cleft also contributes to a reduced serotonergic stress response [190]. Acute amphetamine administration can increase SERT activity at the membrane



[199]. However, repeated administration of amphetamine or its derivatives consistently fails to alter SERT expression or function in the hippocampus [200-205]. Therefore, while psychostimulants interact acutely with SERT, chronic psychostimulant exposure does not appear to alter SERT expression or function in the hippocampus to alter 5-HT activity during withdrawal.

The organic cation transporter 3 (OCT3) is a low affinity, high capacity transporter that contributes to 5-HT clearance, and a high density of OCT3 is present in the hippocampus [206-211]. OCT3 is directly linked to anxiety behavior, as OCT3 knockout mice display an anxiolytic phenotype [212] and OCT3 inhibition has antidepressant-like effects in rats [211]. Similarly, *SERT* knockout mice consistently display heightened OCT3 activity in the hippocampus [213, 214] and increased anxiety-like behavior [215, 216], as well as increased OCT3 mRNA in the hippocampus (but not other brain regions) [214]. This suggests that OCT3 may have a region-specific role for 5-HT reuptake in the hippocampus [210, 212, 214, 217]. Accordingly, amphetamine inhibits OCT3 monoamines transport [209, 218] (although see [219]) and withdrawal from methamphetamine is associated with decreased OCT3 mRNA in *whole brain* homogenates [213]. However, OCT3 expression and function are *increased* in the ventral hippocampus of rats at 24 hours of withdrawal from chronic amphetamine, resulting in increased 5-HT clearance in this region [204, 205]. Thus, psychostimulant exposure may enhance OCT3-mediated serotonin uptake in the hippocampus to produce the heightened anxiety states observed in these animals.

In addition, chronic cocaine administration increases 5HT<sub>1B</sub> autoreceptors [220], which regulate serotonin release and anxiety-like behavior in the ventral hippocampus [221, 222]. Thus, psychostimulant-induced increases of 5HT<sub>1B</sub>- and OCT3 expression in the ventral hippocampus

may reduce ventral hippocampal 5-HT levels and enhance anxiety/avoidance behavior during withdrawal [176, 198, 223-226] (Figure 2). Furthermore, reductions in evoked 5-HT release in the ventral hippocampus have been linked to augmented reinforcing properties of cocaine and ecstasy (MDMA) [227, 228]. Overall, psychostimulant exposure can induce multiple detrimental effects on serotonin signaling during withdrawal that can alter hippocampal activity, disrupt hippocampal communication with reward processing regions (nucleus accumbens), and may culminate in maladaptive behaviors (Figure 2).

The hippocampal *glucocorticoid stress* system may play a key role in anhedonia and dysphoria that drive relapse during psychostimulant withdrawal. In support of this suggestion, major depressive disorder – with core features of anhedonia and dysphoria – is associated with reduced hippocampal GR to MR ratio (GR/MR) [229] and reduced GR expression and function [230-232]. Knocking out central GR expression (except in the hypothalamus) produces a reliable depression-like phenotype in rodents, which is restored with tricyclic antidepressant treatment [233]. Antidepressants also increase hippocampal GR/MR ratio, expression, and function [234-237], and short-term treatment with the GR antagonist mifepristone improves depressive symptoms in hypercortisolemic patients [238, 239].

Repeated psychostimulant exposure – which produces dysphoric states in withdrawal [13, 240-242] – also results in reduced GR expression – and a reduced GR/MR ratio – in the ventral hippocampus (in rats) [114]. The reduced GR/MR ratio may result in MRs having a more pronounced effect in the ventral hippocampus [114], which may function to preserve HPA regulation and homeostasis, since MRs are thought to preserve basal HPA tone [103, 104]. In support of this possibility, neither plasma nor hippocampal corticosterone levels are altered under basal conditions after repeated amphetamine exposure [114]. However, reduced GR/MR

ratio is associated with depression [229], and may thus contribute to the dysphoric states that cause relapse during psychostimulant withdrawal. Further, the reduced GR/MR ratio may alter hippocampal excitability and result in dysregulated serotonin- and dopamine responses to stress (section 2 and [114]).

Interestingly, *protracted* amphetamine withdrawal (2 weeks) results in an enhanced corticosterone stress response in the ventral hippocampus, without altering basal hippocampal or plasma corticosterone levels, or *stress-induced* plasma corticosterone levels [134]. This enhanced hippocampal corticosterone stress response – paired with the possible persistence of lower GR/MR ratio in the ventral hippocampus [114] – may affect hippocampal regulation of accumbal dopamine output and drug salience (section 2 and [29]). For example, preliminary findings suggest that a stress-relevant concentration of corticosterone infused into the ventral hippocampus rapidly enhances accumbal shell dopamine output (section 2 and [29]), which may enable stress to enhance reward value [63] and promote goal-oriented behavior [60]. In amphetamine withdrawal, infusing corticosterone into the ventral hippocampus may *reduce* accumbal dopamine output [29]. Thus, corticosterone in the ventral hippocampus may enable stress to *reduce* reward value during psychostimulant withdrawal, thereby contributing to anhedonia and dysphoria that can prompt relapse [13, 16]. Overall, these recent findings support a role for hippocampal corticosterone in mediating reward responses to stress, and suggest that dysregulated corticosterone signaling in the ventral hippocampus may contribute to stress-induced relapse during psychostimulant withdrawal.

Acute stress exposure has also been found to produce an immediate 3-fold increase of free corticosterone levels in the dorsal hippocampus [243]. GR/MR ratio is also altered in the dorsal hippocampus during psychostimulant withdrawal [114, 243]. In [243] an *increase* in

GR/MR mRNA ratio was observed in the dorsal dentate and CA1 in response to withdrawal from extended access to daily cocaine self-administration, accompanied by increased GR mRNA in the dentate and CA3, and increased MR mRNA in the dentate. In contrast, others have shown that repeated amphetamine administration selectively *down*-regulates GR mRNA in the dorsal hippocampus (when sampled as a whole) [244-247]. Furthermore, in [114] a *reduction* in dorsal hippocampal GR/MR protein ratio was observed in response to repeated amphetamine exposure during acute (24h) withdrawal, even though neither GR nor MR protein expression were significantly reduced [114]. The lack of change in GR protein expression was also observed after cocaine self-administration [248]. These differences suggest a possible dissociation between mRNA and protein expression, and may also suggest that psychostimulant exposure has differential effects on GR/MR regulation, dependent upon the exposure model, duration of drug abstinence, and hippocampal sub-region assessed.

Overall, the effects of psychostimulant exposure in the dorsal hippocampus seem to alter GR/MR protein ratio as well as GR and MR mRNA levels. The reduced GR/MR ratio in the dorsal hippocampus could reduce corticosterone-induced serotonin activity in that region [196], similar to the reduction observed in the ventral hippocampus [114]. If present, reduced corticosterone-induced serotonin activity in the dorsal hippocampus could impair serotonin-mediated processing of stress-related memories [187] and thus disrupt stress adaptation. The resultant reduced stress coping ability could contribute to stress-induced relapse during psychostimulant withdrawal, as has been reported in humans [13]. Furthermore, the dorsal hippocampus sends excitatory projections to the nucleus accumbens core [51], where dopamine release is associated with coordinating motor programs necessary for drug-seeking [63]. However, dorsal hippocampal stimulation reduces extracellular dopamine in the accumbens core

[249] where differential dopaminergic responses are observed in response to appetitive stimuli (increased dopamine) and aversive stimuli (decreased dopamine), while the dopaminergic response in the shell is enhanced regardless of stimulus type [250, 251]. Thus, future research should further dissect the differential roles of the dorsal and ventral hippocampus in contributing to psychostimulant abuse and withdrawal pathology through interactions with the mesolimbic dopamine system and stress responsivity.

#### **4. Psychostimulant regulation of hippocampal structural plasticity: drug-context and negative affect**

Psychostimulants dramatically alter structural plasticity; inducing long term changes to dendrite- and dendritic spine morphology [252], and potentially altering adult neurogenesis, the process by which new neurons are generated in adulthood. Adult neurogenesis enables experience to alter neuronal circuitry (structural plasticity) in the hippocampus and other regions [253-256]. Adult neurogenesis in the dentate gyrus sub-region of the hippocampus, an essential region for drug-reward-memory formation [152], plays a role in hippocampal-dependent learning and memory [257-259], as well as hippocampal regulation of stress responses [260, 261] and anxiety-like behaviors [262].

Learning processes increase long term survival of new neurons [263, 264] and contextual learning and remembering (novel object recognition) depend upon neuron survival for the ability to rearrange circuits (structural plasticity) [265-268]. Interestingly, removing new neurons after contextual fear- or water maze- training degrades memory [269]. However, increasing neurogenesis after training promotes *forgetting* of hippocampal-dependent recent memory, but not remote- or hippocampus-*independent* memory [270, 271]. Thus, augmented hippocampal

neurogenesis can weaken existing memories and facilitate encoding of new experiences, whereas diminished neurogenesis can stabilize existing memories and impede new memory encoding. Similarly, adult neurogenesis promotes cognitive flexibility and inhibitory control, behaviors regulated by the ventral hippocampus, suggesting ventral hippocampal neurogenesis significantly contributes to these behaviors [272-275].

Importantly, dorsal-ventral differences are distinguished in hippocampal neurogenesis processes. Several studies indicate predominant neurogenesis in the *dorsal*- compared to the *ventral*- dentate gyrus [225, 276-280]. However, new neurons mature more slowly in the ventral dentate than in the dorsal, suggesting a prolonged period in which immature neurons could be influenced by activity and incorporated or removed from local circuitry [281, 282]. Therefore, a larger pool of potential new neurons in the *dorsal* dentate gyrus might contribute to rapid spatial memory formation, whereas slower maturation in the *ventral* dentate gyrus may support the regulation of affective states. In support of this notion, an enriched environment preferentially increases neurogenesis in the *dorsal* dentate, whereas antidepressant treatment increases neurogenesis and chronic stress decreases neurogenesis to a greater degree in the *ventral* dentate gyrus [283-287].

The specific role of dentate gyrus neurogenesis in regulating anxiety and negative affect remains unclear [288]. Several studies correlate reduced neurogenesis with increased anxiety-like behaviors [262, 289-291]. For example, antidepressants that reduce anxiety states stimulate neurogenesis in the rodent and human hippocampus [292-295]. However, suppressing neurogenesis alone does not seem to be sufficient to induce anxiety-like behaviors [296-299]. Events that induce negative affect – such as chronic stress – also suppress adult hippocampal neurogenesis [300] and increasing adult neurogenesis reduces anxiety and depression-like

behaviors in mice treated chronically with corticosterone [301], supporting a role for neurogenesis in mediating hippocampal responses to stress. Stress-induced suppression of cell proliferation in the hippocampus may occur through GRs, which are expressed on proliferating cells [302]. Further, impaired neurogenesis is associated with weakened HPA axis feedback inhibition and increased glucocorticoid levels after acute stress [260, 261]. This suggests that neurogenesis may maintain hippocampal regulation of HPA activity. Thus, impaired neurogenesis may intensify subsequent glucocorticoid effects on hippocampal function, in part through altered serotonergic neurotransmission (see section 3). This may induce long-term stress sensitivity and negative affect.

Psychostimulants directly regulate the process of adult hippocampal neurogenesis. In rats, chronic but not acute cocaine exposure reduces proliferation rates in the dentate gyrus, but does not alter newborn cell survival rates [303-305]. However, in mice, cocaine seems to increase proliferation [306], and its effects on neuron survival appear to depend on existing vulnerability and drug dosage [307, 308]. Amphetamines have less of an impact on proliferation rates (relative to cocaine), but a greater tendency to reduce the long-term survival of newborn cells [225, 309, 310]. However, methamphetamine exposure reduces both proliferation and survival of new neurons [311, 312]. While most research has focused on the negative regulation of neurogenesis by drugs of abuse, multiple positive effects on neurogenesis have also been observed, particularly during withdrawal. These include increased markers of immature neurons during withdrawal [305, 306, 313, 314] and increased survival of hippocampal progenitors [315, 316]. It appears that drug-seeking behaviors persist independent of recovery from initial drug-induced decreases in new neuron proliferation [305]. However, altered hippocampal neurogenesis impacts drug-taking behaviors. When hippocampal neurogenesis is impaired prior to cocaine

self-administration training, rats take greater amounts of cocaine and display higher breakpoints (vs controls), suggesting an intensification of drug reward [317]. Natural reward (sucrose administration) is not altered by this process [317], although transgenic mice with impaired neurogenesis exhibit no sucrose preference, which is an indication of anhedonia [261]. Further, impairing neurogenesis prior to cocaine self-administration training does *not* alter relapse to drug-seeking [317], yet impairing neurogenesis *after* self-administration training – or before CPP – increases context-induced drug-seeking behavior and impedes extinction [317, 318]. This suggests that impaired neurogenesis enhances potency of drug-associated environmental cues in a time-dependent fashion, and *enhancing* neurogenesis may promote *forgetting* of recent hippocampal-dependent drug-reward memory [270]. Increased neurogenesis elicited by voluntary wheel-running or environmental enrichment *before* conditioning also delays extinction of cocaine CPP, whereas running that occurs *after* conditioning accelerates cocaine CPP extinction [319, 320] (although see [321]). Together, these studies suggest that hippocampal neurogenesis may play a role in drug reward-context memory formation and relapse to drug-seeking.

Psychostimulants may alter neurogenesis processes at least partially through their interactions with the hippocampal dopamine system. Dopamine is known to selectively modulate neurogenesis and immature neuron activity [322], and the ventral hippocampus receives a higher density of dopaminergic inputs than the dorsal hippocampus [323], which may contribute to the dorsa-ventral differences observed in hippocampal neurogenesis processes (described above). Interestingly, dopamine receptor activation promotes adult hippocampal neurogenesis [324, 325], but dopamine can also decrease the capacity of young neurons to express LTP by persistently attenuating young neuron inputs [322]. Psychostimulant-induced alterations to hippocampal



dopamine output could then selectively modulate the activity of immature neurons and dictate their subsequent integration into hippocampal circuitry. In support of this suggestion, cocaine enhances LTP magnitude selectively in the ventral hippocampus (where dopamine innervation is highest) in a dopamine-receptor-dependent fashion [159]. Likewise, cocaine-induced CPP stimulates context-dependent activation of adult-born neurons to a greater extent in the ventral dentate gyrus [326]. Altogether, these findings suggest that psychostimulants may exert dynamic effects on hippocampal neurogenesis, promoting functional integration or reducing proliferation or survival, depending upon hippocampal region and age of the newly-generated cells at the time of drug experience [327] (Figure 2). This preferential activation could promote formation and incubation of drug-context associations. Additionally, altered neurogenesis – perhaps through changes in immature neurons – could indirectly influence hippocampal networks involved in mediating anxiety states – including those induced by drug use and withdrawal – depending upon individual susceptibility, experience, and withdrawal state (Figure 2). Overall, more studies are necessary to determine the long-term impact of psychostimulants and withdrawal on new neuron integration along the dorsal-ventral extent of the hippocampus. Specifically, it will be important to uncover the subsequent impact of psychostimulant-induced neurogenesis on drug memory reinstatement, and further identify the underlying mechanisms at play, to develop new therapeutic strategies.

## **5. Conclusions**

Together, the literature reviewed indicates that the hippocampus contributes to drug reward processes, drug-related memory formation, and drug-induced anxiety and dysphoria. Neuroadaptations following repeated drug administration lead to heightened hippocampal-

mesoaccumbal activity, thus amplifying responses to psychostimulants and associated cues. At the same time, a persistent dysregulation of the hippocampal component of the brain's emotional system produces a bias towards negative affect-like responses (Figure 2). Moreover, long term alterations of neurogenesis within the hippocampus may contribute to relapse vulnerability through enhanced drug sensitivity, enhanced drug memory, or anxiogenic stimuli. However, further study is necessary to determine how psychostimulants modulate the hippocampus to heighten hippocampal-mesoaccumbal activity, and particularly how hippocampal neurogenesis functions to influence drug reward and drug-related memories. Future studies should also explore the functional implications of the impact of drugs of abuse and withdrawal on the hippocampus regarding its dorsal-ventral axis. A better understanding of regional differences may help clarify the roles of neurogenesis in changes induced by psychostimulants on different types of hippocampus-dependent behavior. Taking into consideration the activity of these hippocampal systems under drug naïve conditions, chronic psychostimulant-induced alterations to the hippocampus produce ineffective maladaptive behavioral responses to stress and environmental challenges. Restoration of these abnormalities within the hippocampus, either in neuronal activity, neurochemical levels, or neurogenesis could provide an effective therapeutic option to ameliorate negative affect and relapse vulnerability in psychostimulant addiction.

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## Chapter 1: Scientific premise

This research focuses on the ability of a peripheral stress hormone (cortisol in humans, corticosterone in rodents) to act as a neuromodulator in the brain. More specifically, this work explores the way corticosterone in the ventral hippocampus (a brain region associated with stress regulation and emotion (Bannerman *et al.*, 2004; Herman & Mueller, 2006; Barr *et al.*, 2017) can interact with the mesolimbic system to alter the reward neurotransmitter dopamine (Baik, 2013) in the nucleus accumbal shell, a brain region associated with reward/incentive salience and motivation (Floresco, 2014; Berridge & Robinson, 2016). This interaction can provide a mechanism that enables stress to enhance reward salience and direct goal-oriented behavior in control conditions (Hollon *et al.*, 2015; Berridge & Robinson, 2016), and induce the dysphoric states that drive relapse during amphetamine withdrawal (Belujon & Grace, 2011; Barr *et al.*, 2017) (in rats).

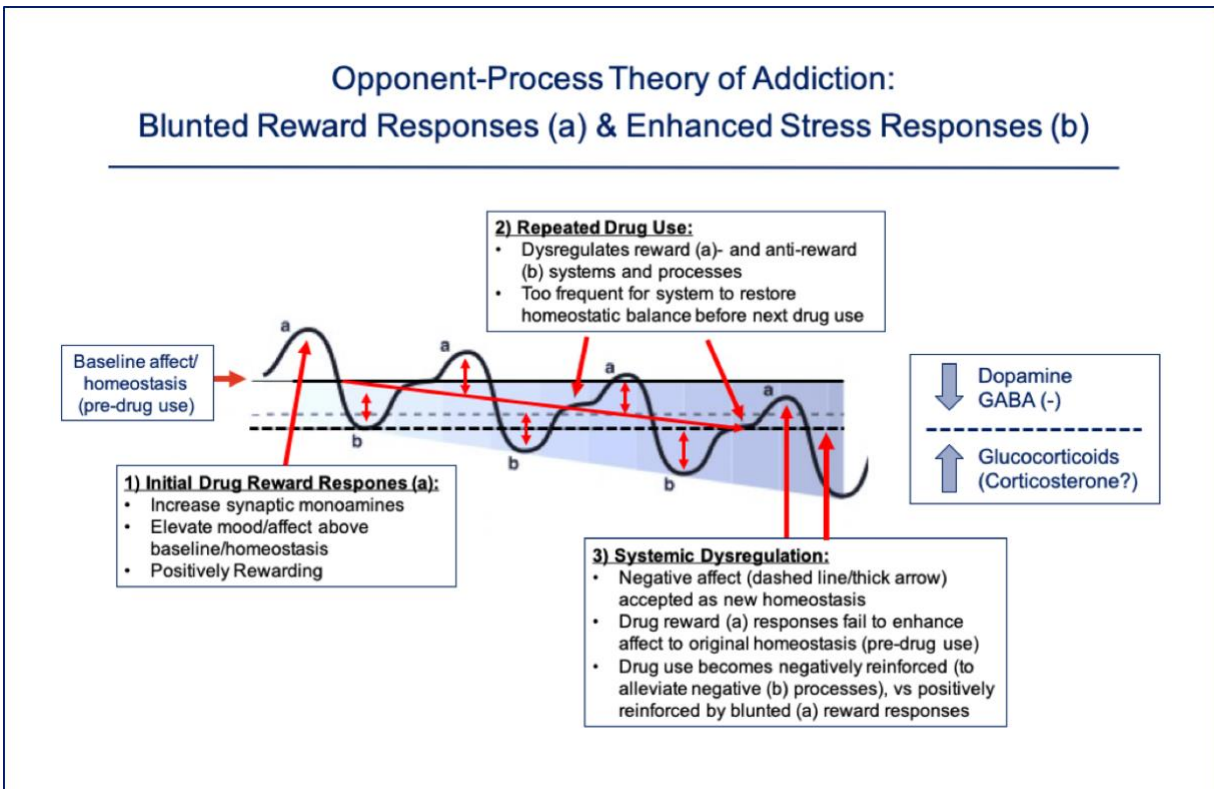
Although stress can feel uncomfortable, it plays an important role in motivating goal-oriented behaviors. This may be through the ability of stress to mediate dopamine reward systems centrally (Hollon *et al.*, 2015). For example, dopamine output in the nucleus accumbens shell enhances reward salience and increases motivational drive (Piazza & Le Moal, 1998; Ganster *et al.*, 2011; Pecina & Berridge, 2013; Pool *et al.*, 2015), and stress is known to increase dopamine levels in the nucleus accumbens shell (but not in the accumbal core) (Kalivas & Duffy, 1995; Enrico *et al.*, 2013). This may provide a mechanism by which stress can enhance motivation and reward value to promote goal-oriented behavior in normative states. However, the specific

mechanisms by which stress enhances accumbal dopamine output, reward salience, and motivation have not been identified in their entirety.

In contrast to the role of stress in healthy conditions, stress plays a very different role in disease states such as amphetamine withdrawal. A variety of preclinical and clinical literature demonstrate that stress exposure, aversive stimuli, and drug stimuli can produce craving (in humans) and negative affect/aversive states (humans and rodents) that are time-locked with immediate *reductions* in accumbal shell dopamine concentrations and frequency of shell dopamine release (in rodents). Moreover, these negative affect states and reductions in accumbal shell dopamine levels directly drive and predict drug-taking behavior (humans and rodents) which alleviates the negative affect (in humans) and immediately restores accumbal shell dopamine levels (in rodents) (Sinha *et al.*, 1999; Sinha *et al.*, 2000; Sinha, 2001; Weise-Kelly & Siegel, 2001; Sinha *et al.*, 2003; Ungless *et al.*, 2004; Sinha *et al.*, 2006; Sinha, 2007; Paliwal *et al.*, 2008; Roitman *et al.*, 2008; Wheeler *et al.*, 2008; Brischoux *et al.*, 2009; Twining *et al.*, 2009; Wheeler & Carelli, 2009; Wheeler *et al.*, 2011; Robinson *et al.*, 2014; Twining *et al.*, 2014). Overall, it appears that stress can *enhance* accumbal shell dopamine output and motivate goal-oriented behavior in normative states (Enrico *et al.*, 2013; Floresco, 2014; Hollon *et al.*, 2015; Berridge & Robinson, 2016). However, stress can *reduce* accumbal shell dopamine output in psychostimulant withdrawal (Roitman *et al.*, 2008; Twining *et al.*, 2014). This reduction in accumbal dopamine release can thereby motivate craving, drug-taking, and relapse during acute and prolonged periods of drug abstinence (Sinha, 2007; Cleck & Blendy, 2008; Paliwal *et al.*, 2008; Wheeler *et al.*, 2008; Wheeler & Carelli, 2009; Koob *et al.*, 2014; Twining *et al.*, 2014; Kwako & Koob, 2017).

Amphetamine-type stimulants include methamphetamine, ecstasy, MDMA, and Adderall (Heal *et al.*, 2013; Uddin *et al.*, 2017). These potent central nervous system stimulants act through a variety of cellular mechanisms to increase synaptic concentrations of the monoamines dopamine, serotonin, and norepinephrine in various mesolimbic brain regions (Heal *et al.*, 2013; Uddin *et al.*, 2017). This can enhance attention and cognition, mood, motivation, and reward (Heal *et al.*, 2013; Uddin *et al.*, 2017). For this reason, amphetamine-type stimulants are used clinically to treat a variety of catecholamine disorders such as attention deficit disorder (ADD) (Fone & Nutt, 2005; Heal *et al.*, 2013; Uddin *et al.*, 2017). For this same reason, they have high potential for abuse (Fone & Nutt, 2005; Heal *et al.*, 2013; Uddin *et al.*, 2017). In fact, amphetamine stimulants are currently the second leading class of abused substances globally behind marijuana (Sun *et al.*, 2014; UNODC, 2016; Winstock *et al.*, 2018). Furthermore, amphetamine use disorder is marked by relapse rates of  $\geq 60\%$  (Brecht *et al.*, 2008; Gonzales *et al.*, 2010; Brecht & Herbeck, 2014), rivaling those of many other commonly abused drugs and chronic illnesses (NIDA, 2018).

Recreational drug use is thought to occur at supratherapeutic doses (Shoptaw *et al.*, 2009; Uddin *et al.*, 2017). *Supratherapeutic* drug use can result in dysregulation of reward and anti-reward neural circuitry mediating a transition from positively reinforced drug-taking to negatively reinforced drug dependence (Fig. 1-1) (Kreek & Koob, 1998; Koob & Kreek, 2007; Koob & Le Moal, 2008a; b; Gardner, 2011; Uddin *et al.*, 2017). This concept is illustrated by the opponent-process theory of addiction, which proposes that repeated drug use blunts positively reinforcing dopamine reward responses and enhances negatively reinforcing “anti-reward” responses (Koob & Kreek, 2007; Koob & Le Moal, 2008a; b).



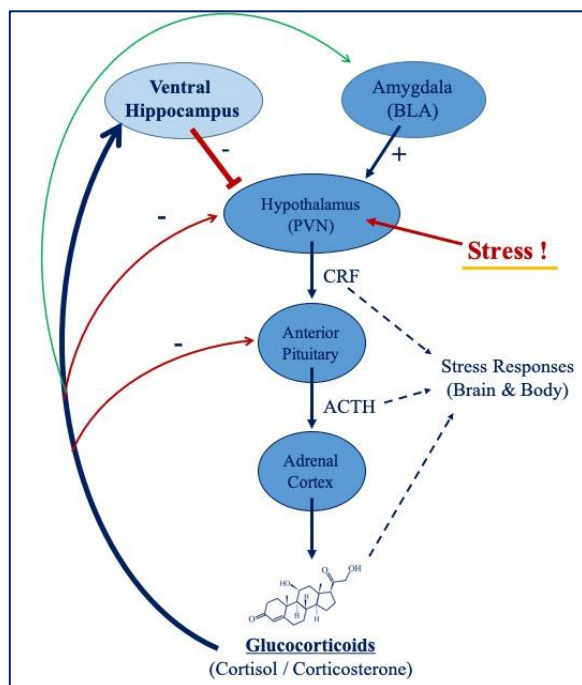
**Figure 1-1. The Opponent-Process Theory of Addiction** suggests repeated drug use at supratherapeutic doses blunts positively reinforcing dopamine reward responses (shown as the “a” processes in the figure) and enhances negatively reinforcing “anti-reward” responses (shown as the “b” process) that are thought to be mediated by the glucocorticoid stress system and can produce anxiogenic and dysphoric states in withdrawal. This homeostatic dysregulation places an overbearing allostatic load on the individual. Over time, the body is unable to restore and maintain homeostatic balance, and a more negative affect is accepted as a new state of homeostasis (#3 and dashed line in the figure). Within this state, it is thought that drug use is no longer positively reinforced, since dopamine reward responses (the “a” processes) are blunted and fail to return the system to its former homeostatic baseline. Rather, drug-taking is thought to be negatively reinforced by alleviation of the negative affective states (the opponent “b” processes) experienced in withdrawal. Figure adapted from Koob, G.F. (2003) *Neuroadaptive mechanisms of addiction: studies on the extended amygdala*. *European Neuropsychopharmacology*, 13, 442-452.

The “anti-reward” responses described in this theory may be mediated by the glucocorticoid stress system and can produce anxiogenic and dysphoric states in withdrawal (Koob & Le Moal, 2005; Gardner, 2011; Koob *et al.*, 2014; Tu *et al.*, 2014; Barr *et al.*, 2017). Over time, the body is unable to maintain homeostatic balance, and a more negative affect is accepted as a new state of homeostasis. Within this state, it is thought that drug use is no longer positively reinforced, since dopamine reward responses are blunted and fail to return the system to its former homeostatic baseline. Rather, drug taking is thought to be negatively reinforced by alleviation of the negative

affective states experienced in withdrawal (Koob, 2015). Thus, it can be important to understand the mechanisms that produce negative affect during acute and protracted drug withdrawal to help prevent relapse.

Amphetamine withdrawal has a clinically defined DSM-V syndrome that has an 87% prevalence among amphetamine users seeking treatment (American Psychiatric Association, 2013; NIDA, 2018; Shoptaw *et al.*, 2009 Uddin *et al.*, 2017). Associated symptoms include dysphoria (lack of motivation/reward), craving, anxiety, and hypersensitivity to stress in humans (Shoptaw *et al.*, 2009; Kosten, 2012; Uddin *et al.*, 2017) and rodents (Cryan *et al.*, 2003; Russig *et al.*, 2006; Li *et al.*, 2014; Tu *et al.*, 2014; Bray *et al.*, 2016; Barr *et al.*, 2017). Amphetamine users attribute these negative affect symptoms to relapse (Gossop, 2009; Shoptaw *et al.*, 2009). Despite the growing problem of psychostimulant abuse, there are currently no FDA-approved pharmacotherapies that are effective in treating amphetamine withdrawal syndrome (Gossop, 2009; Shoptaw *et al.*, 2009; Kishi *et al.*, 2013; Perez-Mana *et al.*, 2013; Hartel-Petri *et al.*, 2017). Current treatment strategies target the monoamine dysregulation and increased anxiety states that occur during withdrawal. However, these approaches fail to prevent relapse (Srisurapanont *et al.*, 2001; Rothman *et al.*, 2008; Kosten, 2012; Heal *et al.*, 2013). Thus, there is an urgent need for novel pharmacotherapeutic treatment targets that can prevent relapse during amphetamine withdrawal (Shoptaw *et al.*, 2009). Identifying these targets requires a greater understanding of the neurobiological processes that mediate the negative affective states thought to prompt relapse during withdrawal.

Enhanced glucocorticoid stress responses are thought to negatively reinforce drug-taking (Koob & Kreek, 2007; Koob & Le Moal, 2008a; b) and may contribute to the negative affect states that drive relapse (Sinha *et al.*, 2006; Wheeler *et al.*, 2008). Therefore, it could be fruitful to better understand how the glucocorticoid stress system becomes dysregulated with repeated amphetamine exposure and withdrawal. At the systemic level, the hypothalamic-pituitary-adrenal axis (HPA) coordinates neuroendocrine stress responses through peripheral release of glucocorticoid hormones (cortisol in humans, corticosterone in rodents) into the bloodstream (Fig. 1-2). Glucocorticoids are lipophilic and readily cross the blood brain barrier to act on central tissues (Ulrich-Lai & Herman, 2009). Among the central tissues that respond to glucocorticoid exposure, the hippocampus is the primary target for glucocorticoid activation in the brain (McEwen *et al.*, 1968). Furthermore, the ventral subiculum of the ventral hippocampus is the primary limbic region that utilizes glucocorticoid feedback to dampen and terminate stress responses (van Haarst *et al.*, 1997; Herman *et al.*, 2003; Barr *et al.*, 2017).



**Figure 1-2. The Hypothalamic-Pituitary-Adrenal Axis (HPA) coordinates neuroendocrine stress responses.** In response to stress, the paraventricular nucleus of the hypothalamus induces a signaling cascade that ultimately stimulates peripheral secretion of glucocorticoid hormones (cortisol in humans, corticosterone in rodents) into the bloodstream. Glucocorticoids are lipophilic and can readily cross the blood brain barrier to act on central tissues. The hippocampus is the primary target for glucocorticoid activation and the ventral hippocampus is thought to be the primary limbic region that utilizes glucocorticoid feedback to inhibit HPA axis activity through activation of its glucocorticoid and mineralocorticoid receptors. This can dampen and terminate stress responses. Note: the ventral hippocampus is thought to induce inhibition onto the HPA axis by exciting inhibitory projections from the Bed Nucleus of the Stria Terminalis (BNST) via the fimbria/fornix (not shown)(Cullinan *et al.*, 1993). Corticosterone is also thought to inhibit HPA activity at the level the PVN, anterior pituitary, and through other limbic regions (ex: medial prefrontal cortex, not shown), and may excite HPA activity through its actions in the basolateral amygdala (BLA) (Herman *et al.*, 2003; Herman *et al.*, 2005; Herman & Mueller, 2006; Herman *et al.*, 2016)

Rats in amphetamine withdrawal demonstrate enhanced behavioral responses to stress (Li et al., 2014). Hypersensitivity to stress is also a prominent feature of amphetamine withdrawal syndrome that is associated with relapse in humans (Sinha et al., 2006; Shoptaw et al., 2009; Kosten, 2012; Uddin et al., 2017). Therefore, dysregulation of glucocorticoid feedback onto the HPA axis (as induced by the ventral hippocampus, in addition to other limbic regions) could mediate stress hypersensitivity in withdrawal. To explore this possibility, a rat model of amphetamine pre-treatment and withdrawal was established in which adult male Sprague-Dawley rats (8 – 10 weeks of age) received daily injections of d-amphetamine (2.5 mg/kg b.w., i.p.) or saline for 2 weeks followed by a 2-week withdrawal period (Li et al., 2014; Tu et al., 2014; Bray et al., 2016; Solanki et al., 2016). This protocol was found to enhance anxiety and behavioral responses to stress (20 min of restraint) in acute and protracted periods of withdrawal (Table A-2)(Barr et al., 2010; Vuong et al., 2010; Li et al., 2014; Reinbold et al., 2014; Tu et al., 2014). Therefore, this model reproduces the anxiety and stress hypersensitivity associated with amphetamine withdrawal syndrome in humans and has face validity (Willner, 1986).

One effect of corticosterone in the ventral hippocampus is that it can act on glucocorticoid receptors to stimulate serotonin release (Fig. 1-4) (Barr & Forster, 2011; Li *et al.*, 2014). Serotonin has an anxiolytic effect in the ventral hippocampus (Tu *et al.*, 2014) and has been linked to positive stress coping (Joca *et al.*, 2007; Li *et al.*, 2014; Tu *et al.*, 2014). Interestingly, the serotonin stress response in the ventral hippocampus is completely abolished in amphetamine withdrawal (Li *et al.*, 2014). This directly contributes to the observed anxiety states (Tu *et al.*, 2014) and may be associated with reduced stress-coping (see review and discussion in Joca *et al.*, 2007; Li *et al.*, 2014; Tu *et al.*, 2014), enhanced stress-induced behavioral arousal (Li *et al.*,



2014), and relapse vulnerability in withdrawal. Serotonin release is induced by the actions of corticosterone in the ventral hippocampus (Barr & Forster, 2011). Therefore, we tested whether stress-induced *corticosterone* in the ventral hippocampus might also be reduced in amphetamine withdrawal, further contributing to the serotonin deficit and enhanced stress-induced behavioral arousal in withdrawal (Li *et al.*, 2014).

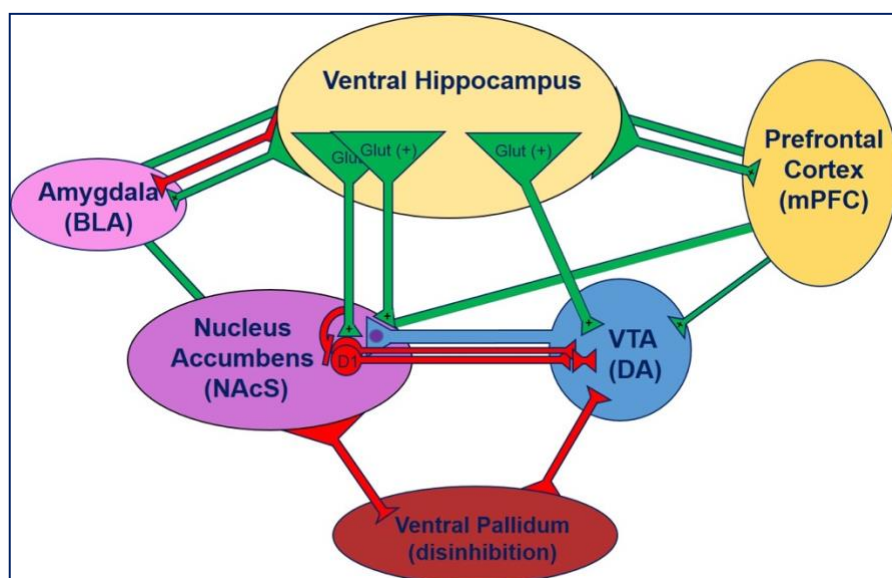
Chapter 2 describes studies designed to explore the effects of repeated amphetamine exposure and withdrawal on the corticosterone stress response both in the ventral hippocampus and peripherally. In these studies, rats were exposed to 20 minutes of restraint stress in the second week of withdrawal from repeated amphetamine (or saline) exposure using the same amphetamine pre-treatment protocol described above. Freely-moving microdialysis was used to collect dialysates from the ventral hippocampus before, during, and after restraint and enzyme-linked immunoassay was used to assess free extracellular corticosterone levels in the dialysates. In a separate group of rats, trunk blood was obtained via rapid decapitation either immediately- or 1 hour after restraint, with plasma samples also obtained from stress-naïve rats in a time-paired fashion and enzyme-linked immunoassay used to assess total-, free-, and bound levels of corticosterone in the plasma.

The second focus of this research was based on the observations that ventral hippocampal corticosterone is known to mediate central-, peripheral-, and behavioral stress responses (Herman & Mueller, 2006; Barr & Forster, 2011; Li *et al.*, 2014; Tu *et al.*, 2014). We set out to explore further possible effects of corticosterone in the ventral hippocampus in chapter 3, as

potentially relative to the stress hypersensitivity, negative affect, and dysphoria thought to drive relapse during withdrawal (Gossop, 2009; Uddin *et al.*, 2017).

Stress and corticosterone are thought to be excitatory in the hippocampus and can potentiate rapid glutamate release *in vitro* and *in vivo* (Bagley & Moghaddam, 1997; Venero & Borrell, 1999; Karst *et al.*, 2005). In the *ventral* hippocampus, excitation (via electrical stimulation or infusion of the glutamate receptor agonist NMDA) can stimulate dopamine release in the nucleus accumbens shell *in vivo* and reinstate extinct amphetamine self-administration (in rats) (Blaha *et al.*, 1997; Barr *et al.*, 2014; Taepavarapruk *et al.*, 2014). This may occur through a variety of mechanisms addressed in Fig. 1-3. Moreover, repeated psychostimulant exposure *enhances* the ability of NMDA infusions in the ventral hippocampus to stimulate accumbal dopamine output during acute (< 24 h) withdrawal (Barr *et al.*, 2014). If corticosterone is indeed excitatory in the ventral hippocampus *in vivo* (Karst *et al.*, 2005; Wang & Wang, 2009), this would suggest that *corticosterone* in the ventral hippocampus could also enhance accumbal dopamine excitation in the nucleus accumbens shell, and this increase may become dysregulated in amphetamine withdrawal. However, whether corticosterone in the ventral hippocampus actually *does* regulate dopamine reward systems has never been directly tested. Therefore, chapter 3 describes studies designed to test whether corticosterone in the ventral hippocampus can alter accumbal dopamine output in control conditions and in amphetamine withdrawal.

We hypothesized that corticosterone in the ventral hippocampus would enhance accumbal dopamine output in control conditions. However, we were curious as to whether ventral hippocampal corticosterone may produce a different effect on accumbal dopamine release in

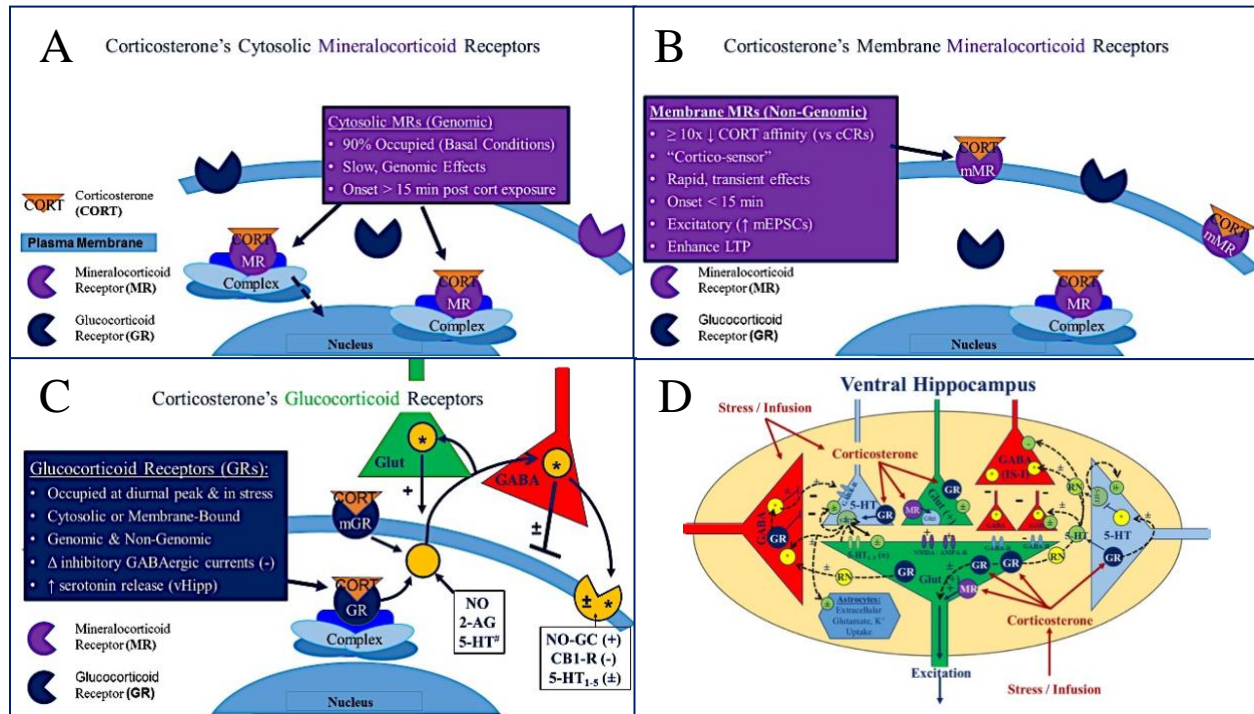


the soma within the VTA and onto the dopamine terminals within the NAcS and can increase cell firing (from the VTA) and stimulate terminal dopamine release (within the NAcS) respectively (Legault et al., 2000; Floresco et al., 2001; Geisler et al., 2007; Valenti et al., 2011; Britt et al., 2012; Tye, 2012). A separate population of glutamatergic efferents from the ventral hippocampus that projects to the NAcS is disynaptic and terminates onto excitatory D1- and inhibitory D2 medium spiny neurons (MSNs) within the NAcS and onto feed-forward interneurons. The interneurons target MSNs and tightly regulate the excitatory/inhibitory balance of MSNs locally and create an overall inhibitory “backdrop” within the NAcS in control conditions (Scudder et al., 2018). Dopamine and MSN excitation in the NAcS can also regulate VTA dopamine output reciprocally through direct and indirect pathways (Floresco et al., 2001; Pignatelli & Bonci, 2018). For example, the NAcS sends inhibitory projections to the ventral pallidum (VP, shown in crimson; inhibitory GABAergic projections shown in red) that can disinhibit VTA activity, and the glutamatergic efferents from the ventral subiculum have been functionally linked to this circuit (Floresco et al., 2001). The NAcS also sends two separate populations of direct GABAergic projections to the medial VTA that respond to accumbal shell dopamine and terminate onto VTA dopamine soma or onto feed-forward inhibitory interneurons in the VTA that project onto the VTA dopamine soma, thus enabling accumbal dopamine output to rapidly induce feedback inhibition- or disinhibition onto the VTA directly (Pignatelli & Bonci, 2018; Yang et al., 2018). Finally, the ventral hippocampus sends glutamatergic and GABAergic projections to the basolateral amygdala (BLA, pink) and medial prefrontal cortex (mPFC, gold), both of which send glutamatergic afferents to the NAcS and VTA that enhance NAcS dopamine release in turn (Imperato et al., 1990; Carr & Sesack, 1996; Kalivas, 2000; Howland et al., 2002; Wanchoo et al., 2009; Britt et al., 2012; Tye, 2012; Tye & Deisseroth, 2012; Floresco, 2014; Strange et al., 2014). The mPFC and amygdala also send excitatory projections to the ventral hippocampus, which can regulate NAcS dopamine output and subsequent behaviors indirectly through the ventral hippocampal inputs (Tye, 2012; Tye & Deisseroth, 2012; Barr et al., 2017). In this figure, green projections represent excitatory glutamatergic projections; red projections represent inhibitory GABAergic projection neurons and interneurons. **Abbreviations:** BLA: Basolateral amygdala; D1: Excitatory type-1 G<sub>s/o</sub> dopamine receptor-expressing medium spiny neuron; DA: Dopamine; Glut: Glutamate; mPFC: Medial prefrontal cortex; NAcS: Nucleus accumbens shell; VTA: ventral tegmental area.

protracted amphetamine withdrawal. For example, repeated cocaine exposure was found to enhance the ability of NMDA infusions in the ventral hippocampus to stimulate accumbal dopamine release in acute (< 24 h) withdrawal (Barr *et al.*, 2014). Furthermore, stress produces differential effects on accumbal shell dopamine release in control conditions and in psychostimulant withdrawal (Kalivas & Duffy, 1995; Tidey & Miczek, 1997; Rouge-Pont *et al.*, 1998; Lillrank *et al.*, 1999; Barrot *et al.*, 2000; Scheggi *et al.*, 2002; Enrico *et al.*, 2013; Twining

*et al.*, 2014). Stress can also increase corticosterone in the ventral hippocampus, which is enhanced in amphetamine withdrawal (Bray *et al.*, 2016). Therefore, studies in chapter 3 were designed to directly test the effects of ventral hippocampal corticosterone on accumbal dopamine release in control and withdrawal conditions. Stress-relevant concentrations of corticosterone or vehicle were infused into the ventral hippocampus of urethane-anesthetized adult male rats in control conditions and in the second week of withdrawal from repeated amphetamine exposure (as described above and in chapter 3). *In vivo* chronoamperometry was used to assess accumbal dopamine output at 30 second intervals before, during, and after infusions.

At the cellular and molecular level, corticosterone can act on two different receptor subtypes: mineralocorticoid and glucocorticoid receptors, both of which are highly expressed in the ventral hippocampus (Reul & de Kloet, 1986; Chao *et al.*, 1989; Herman *et al.*, 1989). These receptors can be genomic or non-genomic, cytosolic or membrane-bound, and can differ in their affinity for corticosterone, downstream signaling mechanisms, and temporal signatures in a regionally-dependent manner (Fig. 1-4) (Groeneweg *et al.*, 2012; Barr *et al.*, 2017; Joels & de Kloet, 2017). Cytosolic mineralocorticoid receptors have a high affinity for corticosterone, and are thus ~90% occupied under basal conditions (Reul & de Kloet, 1986; Chao *et al.*, 1989; Herman *et al.*, 1989; Joels & de Kloet, 2017). They are presumed to act primarily through slower genomic mechanisms and are not traditionally associated with initiating rapid stress responses (Joels & de Kloet, 2017). Membrane mineralocorticoid receptors have a 10-fold lower affinity for corticosterone than their cytosolic counterparts and are thought to act through rapid non-genomic mechanisms to potentiate excitatory glutamate release in response to stress (Karst *et al.*, 2005; Maggio & Segal, 2007; Groeneweg *et al.*, 2011; 2012; Joels & de Kloet, 2017).



**Figure 1-4. Corticosterone's Mineralocorticoid and Glucocorticoid Receptors.** Corticosterone can act on mineralocorticoid (MR) or glucocorticoid (GR) receptors in the ventral hippocampus that can be genomic or non-genomic, cytosolic or membrane-bound (mMR/mGR), and can differ in their affinity for corticosterone, downstream signaling mechanisms, and temporal signatures in a regionally-dependent manner. **A) Cytosolic mineralocorticoid receptors (MRs)** have a high affinity for corticosterone, and are ~90% occupied under basal conditions; they are thought to be primarily genomic and are not traditionally associated with initiating rapid stress responses. **B) Membrane mineralocorticoid receptors (mMRs)** have a 10-fold lower affinity for corticosterone than their cytosolic counterparts and are thought to act through rapid non-genomic mechanisms to potentiate excitatory glutamate release in response to stress (Karst et al., 2005). **C) Non-genomic glucocorticoid receptors (GRs)** also respond to stress-relevant concentrations of corticosterone but can exist in the cytosol or at the membrane. One way postsynaptic non-genomic GRs mediate rapid stress responses is through preferential regulation of interneuron inhibition that involves retrograde neurotransmission (RN). Non-genomic GRs can stimulate synthesis or release of nitric oxide (NO), the endocannabinoid 2-arachidonoylglycerol (2-AG), or serotonin (5-HT) that can interact with NO-sensitive guanylyl cyclase (NO-GC), inhibitory Type I cannabinoid receptors (CB1), or excitatory and inhibitory serotonin receptors expressed on specific subpopulations of GABAergic interneurons to augment, inhibit, or disinhibit presynaptic interneuron activity (see also Fig. 1-4D). Membrane glucocorticoid receptors (mGRs) have also been identified that can potentiate glutamate release (Wang & Want, 2009). However, the extreme amounts of corticosterone required to induce mGR-mediated effects may lack physiological relevance. **D) Receptor Mechanisms that Enable Corticosterone to Mediate Excitation/Inhibition in the Ventral Hippocampus.** A variety of literature suggests that non-genomic membrane mineralocorticoid receptors (MRs, shown in purple) have an excitatory effect in the hippocampus and can rapidly and reversibly potentiate excitatory glutamate release in response to stress. In contrast, non-genomic glucocorticoid receptors (GRs, shown in navy blue) can exist in the membrane or in the cytosol and can tightly regulate local and extrahippocampal excitation and inhibition by induction of multiple signaling pathways and highly selective interactions within the ventral hippocampus' complex interneuronal network (shown in red). Studies from a variety of limbic tissues demonstrate that glucocorticoid receptor activation can increase inhibitory postsynaptic currents in the ventral hippocampus (in vitro) and can recruit rapid serotonin (5-HT), nitric oxide (NO), and endocannabinoid 2-arachidonoylglycerol (2-AG) signaling in the ventral hippocampus and other regions (represented in the figure by yellow and light green retrograde neurotransmission "RN" molecules). These signaling mechanisms can act on inhibitory and excitatory serotonin receptors (shown as light green circles), NO-sensitive guanylyl cyclase (NO-GC, shown as yellow circles with asterisks), and inhibitory type I cannabinoid (CB1) receptors (shown as yellow circles with asterisks) to alter glutamate, GABA, and/or monoaminergic release, depending on the cell target. Serotonin's inhibitory and excitatory receptors demonstrate promiscuous expression on a variety of serotonin terminals (shown in light blue), astrocytes and microglia (light blue hexagon), pyramidal cells, and GABAergic interneurons (shown in red) and long-range projection neurons in the hippocampus. In contrast, excitatory 5-HT<sub>3</sub> receptor expression seems to be confined to interneurons of almost all varieties, including interneuron-specific interneurons (IS-Is) (shown in red), some of which may also express excitatory NO-GC and inhibitory CB1 receptors (shown in yellow). See text for relevant citations. **Abbreviations:** 2-AG: 2-arachidonoylglycerol; 5-HT (±): Serotonin signaling component (terminals shown in light blue; signaling molecules and receptors shown in light green); 5-HT<sub>3</sub>: Excitatory serotonin cation channel receptor; CB1:

*Inhibitory G<sub>i/o</sub>-coupled type I cannabinoid receptor; CORT: Corticosterone; GABA (-): Inhibitory GABA signaling component (neurons shown in red, GABA receptors shown in blue, molecules shown as “-”); Glut (+): Excitatory glutamate signaling component (terminals shown in green, AMPA and NMDA glutamate receptors shown in purple, molecules shown as “+”); GR: Corticosterone’s glucocorticoid receptors (shown in navy blue); mGR/MR: Membrane-associated GR/MR; MR: Corticosterone’s mineralocorticoid receptors (shown in purple); RN: Retrograde neurotransmitter (representative of GR-mediated NO, 2-AG, and serotonin signaling, shown in yellow and yellow/green circles).*

Corticosterone’s low-affinity non-genomic glucocorticoid receptors also respond to stress-relevant concentrations of corticosterone. However, in contrast to membrane mineralocorticoid receptors, a variety of literature suggests non-genomic glucocorticoid receptors can exist in the cytosol *or* at the membrane, and can produce a variety of regionally-specific effects that include tight regulation of local excitation and inhibition through induction of multiple signaling pathways and highly selective interactions with the ventral hippocampus’ complex interneuronal network (as depicted in Figs. 1-4 C – D) (Zeise *et al.*, 1992; Freund & Buzsaki, 1996; Di *et al.*, 2003; Freund *et al.*, 2003; de Kloet *et al.*, 2005; Tasker *et al.*, 2006; Maggio & Segal, 2007; 2009; Hu *et al.*, 2010; Barr & Forster, 2011; Chamberland & Topolnik, 2012; Hill & Tasker, 2012; Di *et al.*, 2016; Barr *et al.*, 2017).

For example, glucocorticoid receptor activation can increase inhibitory postsynaptic currents in the ventral hippocampus, peaking at 45 – 60 min *in vitro* (Maggio & Segal, 2009). Studies from a variety of limbic tissues suggest this may occur through the ability of glucocorticoid receptors to recruit rapid serotonin, nitric oxide (NO), and endocannabinoid 2-arachidonoylglycerol (2-AG) signaling in the ventral hippocampus (for serotonin, shown *in vivo*; Barr & Forster, 2011), dorsal hippocampus (for NO, shown *in vitro*; Hu *et al.*, 2010), and amygdala and hypothalamus (for 2-AG, shown *in vitro*; Di *et al.*, 2003; 2016). These signaling mechanisms can act on inhibitory and excitatory 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>2</sub>, and 5-HT<sub>3</sub> receptors, NO-sensitive guanylyl

cyclase (NO-GC), and inhibitory type I cannabinoid (CB1) receptors (respectively) to alter glutamate, GABA, and/or monoaminergic release depending on the cell target.

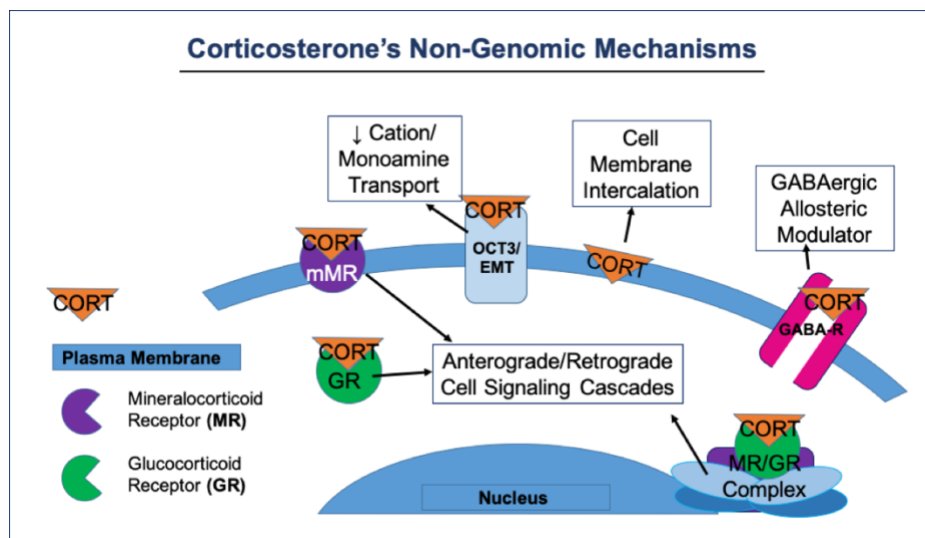
Regarding glucocorticoid receptor recruitment of *serotonin* signaling, serotonin's inhibitory and excitatory receptors demonstrate promiscuous expression on nearly all cell types in the ventral hippocampus, including serotonin terminals, astrocytes and microglia, pyramidal cells, and GABAergic interneurons and long-range projection neurons in the hippocampus (Fig. 1-4D) (Berumen *et al.*, 2012). Moreover, the ventral hippocampus is one of two brain regions with interneurons that specifically target other interneurons (appropriately termed *interneuron-specific interneurons*, IS-Is) (Freund & Buzsaki, 1996; Isaacson & Scanziani, 2011; Chamberland & Topolnik, 2012). In contrast to serotonin's other excitatory and inhibitory receptors, serotonin's excitatory 5-HT<sub>3</sub> receptor expression seems to be confined to interneurons of almost all varieties, including IS-Is (Freund & Buzsaki, 1996; Berumen *et al.*, 2012; Chamberland & Topolnik, 2012; Pelkey *et al.*, 2017). This suggests that glucocorticoid receptors may tightly regulate local and extrahippocampal excitation and inhibition at least partly through their recruitment of serotonin signaling, which can regulate interneuron activity and stimulate IS-I disinhibition (Fig. 1-4). Therefore, we were curious as to whether the inability of glucocorticoid receptors to recruit serotonin signaling during amphetamine withdrawal (Barr & Forster, 2011; Li *et al.*, 2014) may result in glucocorticoid receptor activation having different effects on excitation and inhibition of accumbal shell dopamine release during withdrawal. Therefore, chapter 3 also describes studies designed to test whether independently activating either glucocorticoid or mineralocorticoid receptors would reveal different effects on accumbal dopamine output in control and withdrawal conditions.

Furthermore, corticosterone's rapid responses to stress are typically thought to be mediated by non-genomic mechanisms (de Kloet *et al.*, 2008; Groeneweg *et al.*, 2011; McEwen *et al.*, 2016). These non-genomic effects have traditionally been associated with activation of mechanisms localized to the cell membrane (Fig. 1-5). However, *cytosolic* mechanisms *can* also induce non-genomic effects (Tumlin *et al.*, 1997; Croxtall *et al.*, 2002; Liu *et al.*, 2010). Therefore, chapter 3 also describes studies designed to test whether the ability of ventral hippocampal corticosterone to alter accumbal dopamine output can be isolated to membrane-associated mechanisms or requires cytosolic access.

We projected that selectively blocking either glucocorticoid receptors, mineralocorticoid receptors, or corticosterone's access to cytosolic mechanisms could isolate corticosterone's effects to one particular receptor or to the cell membrane. We hoped this technique would separately reveal the functional effects of each of the two receptor subtypes on accumbal dopamine output and illuminate whether these effects are driven by mechanisms localized to the cell membrane. We thought that membrane mineralocorticoid receptors would be excitatory in the ventral hippocampus, as observed by others in hippocampal tissues *in vitro* (Karst *et al.*, 2005; Maggio & Segal, 2007). Therefore, we hypothesized that an excitatory effect of ventral hippocampal corticosterone on accumbal dopamine output may be observed, even with glucocorticoid receptors or membrane passage blocked. Glucocorticoid receptors seem to tightly regulate local and extrahippocampal excitation and inhibition with sophisticated specificity that involves glucocorticoid receptor recruitment of serotonin signaling (Fig. 1-4). Therefore, we thought the inability of glucocorticoid receptors to recruit serotonin signaling in amphetamine withdrawal (Barr & Forster, 2011; Li *et al.*, 2014) may provide a mechanistic explanation for the



differential effects of ventral hippocampal corticosterone on accumbal dopamine release in control conditions and in protracted withdrawal (Fig. 3-3A). Thus, we hypothesized that blocking mineralocorticoid receptors may reveal differential effects of glucocorticoid receptor activation in the ventral hippocampus on accumbal dopamine release in control conditions and in protracted withdrawal.



**Figure 1-5. Corticosterone's Non-Genomic Mechanisms** can include: activation of membrane-bound glucocorticoid- and mineralocorticoid receptors (Groeneweg et al., 2012; Barr et al., 2017; Joels & de Kloet, 2017) and inhibition of monoamine transporters such as the organic cation transporter 3 (OCT3) and the extracellular monoamine transporter (EMT) responsible for serotonin and norepinephrine reuptake respectively (Grundemann et al., 1998; Wu et al., 1998; Horvath et al., 2001; Horvath et al., 2003; Gasser, 2006;

Gasser & Lowry, 2018); and positive allosteric modulation of GABA receptors (Orchinik et al., 2001; Henderson, 2007; Volkova et al., 2016). Corticosterone can also become embedded in the cell membrane (independent of receptor binding), which can reduce the functionality of ion channels, transporters, and receptors that enable membrane transport and cellular activity (Buttgereit, 2000; Makara & Haller, 2001; Horvath & Wanner, 2006). Corticosterone's cytosolic receptors can also induce non-genomic effects. For example, corticosterone activation of cytosolic non-genomic glucocorticoid receptors can rapidly and reversibly inhibit ATP-induced currents in rat dorsal root ganglion cultures (Liu et al., 2008) and corticosterone interaction with cytosolic glucocorticoid- and mineralocorticoid receptor complexes can induce dissociation of trans-activator proteins from the cytosolic complex(es) that can then interact with other signaling pathways to rapidly alter signaling cascades in vitro and ex vivo (Tumlin et al., 1997; Croxtall et al., 2002; Horvath & Wanner, 2006).

To test these hypotheses, we selectively blocked either glucocorticoid- or mineralocorticoid receptors in the ventral hippocampus by infusing selective doses of either mifepristone (at a dose for blocking glucocorticoid receptors) (Heikinheimo, 1997; Mahajan & London, 1997; Barr & Forster, 2011) or the mineralocorticoid receptor antagonist spironolactone (Garthwaite & McMahon, 2004) into the ventral hippocampus ten minutes prior to infusing a stress-relevant concentration of corticosterone into the same region of saline- and amphetamine pre-treated rats.

We also infused membrane-impermeable corticosterone (Corticosterone 3-CMO : BSA, or its vehicle) into the ventral hippocampus of a separate group of saline pre-treated rats, thus isolating corticosterone's effects to the cell membrane and preventing access to cytosolic mechanisms (Morozov *et al.*, 1988; Groeneweg *et al.*, 2011). Overall, the experiments in this thesis test the functional ability of the stress hormone corticosterone to act as a neuromodulator in the ventral hippocampus and alter reward neurotransmission. These experiments also test the overall goal of understanding how repeated amphetamine exposure alters the ventral hippocampal glucocorticoid stress system during protracted periods of withdrawal.

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**Chapter 2: Differential effects of amphetamine withdrawal on central and peripheral corticosterone levels in response to stress.**

**Abstract**

Amphetamine withdrawal is associated with heightened anxious behavior, which is directly driven by blunted stress-induced glucocorticoid receptor-dependent serotonin release in the ventral hippocampus as mediated by glucocorticoid receptors. This suggests that glucocorticoid availability in the ventral hippocampus during stress may be reduced during amphetamine withdrawal. Therefore, we tested whether amphetamine withdrawal alters either peripheral or hippocampal corticosterone stress responses. Adult male rats received amphetamine (2.5 mg/kg, i.p.) or saline for 14 days followed by 2 weeks of withdrawal. Contrary to our prediction, microdialysis samples from freely-moving rats revealed that restraint stress-induced corticosterone levels in the ventral hippocampus are enhanced by amphetamine withdrawal relative to controls. In separate groups of rats, plasma corticosterone levels increased immediately after 20 minutes of restraint and decreased to below stress-naïve levels after 1 hour, indicating negative feedback regulation of corticosterone following stress. However, plasma corticosterone responses were similar in amphetamine-withdrawn and control rats. Neither amphetamine nor stress exposure significantly altered protein expression or enzyme activity of the steroidogenic enzymes 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD1) or hexose-6-phosphate dehydrogenase (H6PD) in the ventral hippocampus. Our findings demonstrate for the

first time that amphetamine withdrawal potentiates stress-induced corticosterone in the ventral hippocampus, which may contribute to increased behavioral stress sensitivity previously observed during amphetamine withdrawal. However, this is not mediated by changes in plasma corticosterone or hippocampal steroidogenic enzymes. Establishing enhanced ventral hippocampal corticosterone as a direct cause of greater stress sensitivity may identify the glucocorticoid system as a novel target for treating behavioral symptoms of amphetamine withdrawal.

## **1. Introduction**

Amphetamine dependence is a global health problem with a high incidence of relapse and few successful treatment options (Fone & Nutt, 2005; Wilens *et al.*, 2008; Pomerleau *et al.*, 2012; Heal *et al.*, 2013). Amphetamine withdrawal is associated with anxiety and hypersensitivity to stressors in humans (Cleck & Blendy, 2008; Shoptaw *et al.*, 2009) and rodents (Russig *et al.*, 2006; Barr *et al.*, 2010; Vuong *et al.*, 2010; Li *et al.*, 2014; Tu *et al.*, 2014) that can induce relapse in humans (Gossop, 2009) and maintains the cycle of addiction (Shoptaw *et al.*, 2009; Koob *et al.*, 2014).

Stress induces serotonin release in the ventral hippocampus, which has been implicated in reducing anxiety and stress responsiveness (Graeff *et al.*, 1996; Herman *et al.*, 2003; Li *et al.*, 2014; Tu *et al.*, 2014). We have previously found that rats in the second week of withdrawal from repeated amphetamine exposure show heightened behavioral anxiety (Barr *et al.*, 2010; Vuong *et al.*, 2010; Reinbold *et al.*, 2014; Tu *et al.*, 2014), enhanced behavioral measures of stress-induced arousal (Li *et al.*, 2014), and severely blunted stress-induced serotonin release in the ventral hippocampus (Li *et al.*, 2014), which is known to cause increased behavioral anxiety

(Tu *et al.*, 2014). Therefore, it is important to understand the mechanism by which amphetamine withdrawal alters stress-induced serotonin levels in the ventral hippocampus.

One mechanism by which amphetamine withdrawal could alter stress-related serotonin function in the ventral hippocampus is via glucocorticoid actions in this region. Stress-induced serotonin release in the ventral hippocampus is mediated by corticosterone activation of local glucocorticoid receptors (Barr & Forster, 2011; Li *et al.*, 2014). Amphetamine withdrawal causes a reduction in ventral hippocampus glucocorticoid receptor expression (Barr & Forster, 2011), which may partly explain the corresponding dampening of stress-evoked serotonin release observed in withdrawn rats (Li *et al.*, 2014). However, glucocorticoid receptor expression is not totally abolished following amphetamine withdrawal, but only reduced by ~30% compared to controls, while mineralocorticoid receptor expression remains unaltered. This raises the possibility that other mechanisms regulating glucocorticoid availability during stress are affected by amphetamine withdrawal to account for blunted glucocorticoid receptor-dependent increases in serotonin release. For instance, stress-induced corticosterone levels in the dorsal hippocampus have been found to increase up to 200% from baseline in drug-naïve rats (Keeney *et al.*, 2006; Droste *et al.*, 2008). Therefore, it is conceivable that stress-induced levels of corticosterone in the ventral hippocampus are reduced or absent during amphetamine withdrawal, lowering corticosterone availability and contributing to the lack of glucocorticoid receptor-dependent stress-induced serotonin release in this region (Li *et al.*, 2014) and to the resultant heightened anxiety states (Tu *et al.*, 2014). This possibility was addressed by the current study, using the same amphetamine treatment and withdrawal regime previously shown to produce heightened behavioral anxiety (Barr *et al.*, 2010; Vuong *et al.*, 2010; Reinbold *et al.*, 2014; Tu *et al.*, 2014),

enhanced stress-induced behavioral arousal (Li *et al.*, 2014), and blunted stress-induced serotonin release in the ventral hippocampus (Li *et al.*, 2014).

Corticosterone is secreted primarily from the adrenal cortex in the periphery and readily crosses the blood brain barrier to act on target tissues, including the hippocampus (Robel & Baulieu, 1994; Pura & Kreze, 2005). Amphetamine withdrawal does not alter basal plasma corticosterone levels relative to saline controls at either 24 hours or 4 weeks of withdrawal (Barr *et al.*, 2010), but it is unknown whether stress-induced plasma corticosterone responses are affected. Therefore, we also tested whether amphetamine withdrawal is associated with reduced stress-induced corticosterone levels in the plasma to explain any alterations in ventral hippocampus concentrations.

Glucocorticoid activation of target tissues can also be regulated at the cellular level by extra-adrenal synthesis (Harris *et al.*, 2001) by enzymes such as 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1), which reduces inert 11-dehydrocorticosterone (11-DHC) to active corticosterone (Harris *et al.*, 2001; Taves *et al.*, 2011). Of the intracellular enzymes that regulate steroidogenesis locally in central tissues including the hippocampus (Seckl, 1997; Harris *et al.*, 2001; Taves *et al.*, 2011), 11 $\beta$ -HSD1 appears to play a major role in stress-induced alterations of hypothalamic-pituitary-adrenal output regulation (Edwards *et al.*, 1988; Harris *et al.*, 2001; Atanasov *et al.*, 2004; Muller *et al.*, 2006; Wyrwoll *et al.*, 2011; Odermatt & Kratschmar, 2012; Ergang *et al.*, 2014; Vodicka *et al.*, 2014). For example, 11 $\beta$ -HSD1 mRNA expression was increased in the ventral CA1 hippocampus following a variable 3-day stress protocol (Ergang *et al.*, 2014) and following a resident intruder paradigm of repeated social stress (Vodicka *et al.*, 2014), suggesting that stress may exert its effects on ventral hippocampus corticosterone availability by altering local 11 $\beta$ -HSD1 activity. Another enzyme, hexose 6

phosphate dehydrogenase (H6PD), directly interacts with 11 $\beta$ -HSD1 in central tissue to stabilize 11 $\beta$ -HSD1 reductase activity (White *et al.*, 2007), regulating 11 $\beta$ -HSD1-imposed glucocorticoid activation in peripheral and central tissues (Hewitt *et al.*, 2005; White *et al.*, 2007). Increased H6PD production contributes to 11 $\beta$ -HSD1 up-regulation of glucocorticoids in liver tissues (Wang *et al.*, 2011) but the role of H6PD expression in central glucocorticoid activation is largely unknown (Wang *et al.*, 2011). Therefore, we also tested the hypothesis that amphetamine exposure alters expression and/or activity of either 11 $\beta$ -HSD1 or H6PD in the ventral hippocampus to alter stress-induced levels of corticosterone during amphetamine withdrawal.

## **2. Results**

### *2.1 Experiment 1 – Amphetamine withdrawal enhances stress-induced corticosterone levels in the ventral hippocampus*

#### *2.1.1 Microdialysis probe placements and baseline corticosterone levels in the ventral hippocampus*

Representative placements of probe membrane surfaces for the ventral hippocampus are drawn to scale and illustrated in Fig. 2-1A. Probe placements were similar between saline and amphetamine pretreated rats, and baseline levels of corticosterone also did not differ between saline (1.83  $\pm$  0.07 ng/ml) and amphetamine (1.65  $\pm$  0.22 ng/ml) pretreatment ( $t_{(11)} = 0.717$ ,  $P = 0.488$ ). Data from rats where the probe missed the ventral hippocampus were excluded from the subsequent analyses.



### *2.1.2 Stress-induced corticosterone in the ventral hippocampus*

Amphetamine-pretreated rats undergoing withdrawal exhibited increased restraint-induced corticosterone in the ventral hippocampus (Fig. 2-1B) with a maximal post-stress average of 3.69  $\pm$  0.74 ng/ml at 20 minutes post-stress. Two-way repeated measure ANOVA revealed significant effects of time ( $F_{(9,96)} = 4.175$ ,  $P < 0.001$ ) and an interaction between treatment and time ( $F_{(9,96)} = 2.005$ ,  $P = 0.047$ ) on corticosterone levels. There was no effect of stress over time in saline rats ( $F_{(9,43)} = 0.878$ ,  $P = 0.552$ ) with maximal levels of corticosterone of 2.63  $\pm$  0.39 ng/ml measured 20 minutes post-stress. However, an effect of stress over time was observed for amphetamine-pretreated rats ( $F_{(9,53)} = 4.267$ ,  $P < 0.001$ ) that was apparent at 20 minutes post-stress as compared to pre-stress levels (Holm-Sidak  $P = 0.002$ ). Ventral hippocampus corticosterone was significantly higher in amphetamine pretreated rats as compared to saline controls immediately following restraint stress (SNK,  $P = 0.010$ ) and 20 minutes later (SNK,  $P = 0.006$ ) (Fig. 2-1B).

## *2.2 Experiment 2 – Mechanisms mediating enhanced corticosterone in the ventral hippocampus during amphetamine withdrawal*

### *2.2.1 Amphetamine withdrawal does not alter stress-induced corticosterone levels in the plasma*

A significant effect of stress was observed on total ( $F_{(2, 62)} = 88.427$ ,  $P < 0.001$ ; Fig. 2-2A), free ( $F_{(2, 61)} = 189.847$ ,  $P < 0.001$ ; Fig. 2-2B), and bound ( $F_{(2, 60)} = 11.702$ ,  $P < 0.001$ ; Fig. 2-2C) plasma corticosterone levels. However, no significant effects of amphetamine or saline pretreatment were observed on any measure of plasma corticosterone (total:  $F_{(1, 62)} = 0.030$ ,  $P = 0.862$ ; free:  $F_{(1, 61)} = 0.322$ ,  $P = 0.572$ ; bound:  $F_{(1, 60)} = 0.376$ ,  $P = 0.542$ ). There was no significant interaction present between pretreatment and stress in any of the three measures

(total:  $F_{(2, 62)} = 0.218$ ,  $P = 0.805$ ; free:  $F_{(2, 61)} = 0.028$ ,  $P = 0.972$ ; bound:  $F_{(2, 60)} = 0.574$ ,  $P = 0.566$ ) (Fig. 2-2).

*Post hoc* analysis demonstrated that 20 minutes of restraint stress resulted in higher total (SNK,  $P < 0.001$ ; Fig. 2-2A), free (SNK,  $P < 0.001$ ; Fig. 2-2B), and bound (SNK,  $P = 0.014$ ; Fig. 2-2C) plasma corticosterone levels immediately following restraint relative to stress-naïve levels, and relative to all measures of plasma corticosterone 1 hour following restraint (total: SNK,  $P < 0.001$ ; free: SNK,  $P < 0.001$ ; bound: SNK,  $P < 0.001$ ; Fig. 2-2). All measures of plasma corticosterone levels 1 hour following restraint were also decreased relative to levels observed in stress-naïve controls (total: SNK,  $P = 0.008$ ; free: SNK,  $P = 0.012$ ; bound: SNK,  $P = 0.016$ ).

### *2.2.2 Amphetamine withdrawal does not alter expression of 11 $\beta$ -HSD1 or H6PD in the ventral hippocampus*

Neither amphetamine pretreatment nor stress exposure altered protein expression of 11 $\beta$ -HSD1 or H6PD in the ventral hippocampus during withdrawal (Fig. 2-3). Specifically, protein expression of 11 $\beta$ -HSD1 in the ventral hippocampus was not significantly affected by pretreatment ( $F_{(1, 54)} = 0.001$ ,  $P = 0.977$ ) or restraint stress ( $F_{(2, 54)} = 0.301$ ,  $P = 0.741$ ), and there was no significant interaction between the two factors ( $F_{(2, 54)} = 0.309$ ,  $P = 0.735$ ; Fig. 2-3A). Similarly, protein expression of H6PD in the ventral hippocampus was not significantly altered by pretreatment ( $F_{(1, 51)} = 1.430$ ,  $P = 0.237$ ) or stress ( $F_{(2, 51)} = 2.230$ ,  $P = 0.118$ ), and there was no significant interaction between pretreatment and stress ( $F_{(2, 51)} = 1.153$ ,  $P = 0.324$ ; Fig. 2-3B).

### *2.2.3 Amphetamine withdrawal does not alter activity of 11 $\beta$ -HSD1 in the ventral hippocampus*

Neither amphetamine pretreatment nor stress exposure affected 11 $\beta$ -HSD1 activity or

corticosterone tissue concentration in the ventral hippocampus (Fig. 2-4). Specifically, corticosterone generated *in vitro* within ventral hippocampus tissue in the presence of its precursor 11-DHC (as a measure of 11 $\beta$ -HSD1 activity) was at least 600% greater than corticosterone levels from samples in which 11-DHC was absent, but this increase was not significantly affected by pretreatment ( $F_{(1, 46)} = 1.237$ ,  $P = 0.272$ ) or stress ( $F_{(2, 46)} = 2.038$ ,  $P = 0.142$ ), and there was no significant interaction between the two factors ( $F_{(2, 46)} = 0.0898$ ,  $P = 0.914$ ; Fig. 2-4).

### **3. Discussion**

#### *3.1 Amphetamine withdrawal enhances stress-induced corticosterone levels in the ventral hippocampus*

Given that corticosterone increases serotonin levels in the ventral hippocampus during stress via local glucocorticoid receptor activation (Barr & Forster, 2011), and stress-induced serotonin release in the ventral hippocampus is almost absent during amphetamine withdrawal (Li *et al.*, 2014), we predicted that withdrawal from chronic amphetamine would result in decreased restraint-induced corticosterone in the ventral hippocampus. Instead, we observed an increase in stress-induced corticosterone levels in the ventral hippocampus during amphetamine withdrawal when compared to saline pretreated controls. The reduced stress-induced serotonergic response observed during amphetamine withdrawal, using the same stress paradigm as used here (Li *et al.*, 2014), may instead be explained by a combination of reductions in ventral hippocampus glucocorticoid receptor levels (Barr & Forster, 2011) and increased extracellular serotonin reuptake (Barr *et al.*, 2013), both of which have been observed in the ventral hippocampus following the same amphetamine treatment and withdrawal paradigm used here. It is also

possible that surgical and microdialysis procedures may have altered glucocorticoid function in the ventral hippocampus. However, this possibility would be similar in saline and amphetamine-treated rats, since both groups in this and previous work (Li *et al.*, 2014) underwent probe implantation and dialysis.

In the present study, 20 minutes of novel restraint stress exposure was found to increase free corticosterone levels in the ventral hippocampus by approximately 53% of baseline measures in saline pretreated controls, although this increase was not found to differ significantly from pre-stress levels. In contrast, forty minutes of exposure to a novel environment results in an immediate 3-fold increase in free corticosterone levels in the *dorsal* hippocampus (Droste *et al.*, 2008), and 20 minutes of restraint stress increases corticosterone levels by 200% within the ventral hippocampus in male Wistar rats 6 months of age (Garrido *et al.*, 2012). The discrepancy between the current and previous findings may result from variation in the type of stressor used or the age of the rats tested.

Overall, increased stress-induced corticosterone in the ventral hippocampus during amphetamine withdrawal may have consequences for the associated heightened anxiety and stress responsiveness (Vuong *et al.*, 2010; Li *et al.*, 2014). Amphetamine withdrawal is associated with a reduced glucocorticoid receptor to mineralocorticoid receptor ratio in the ventral hippocampus (Barr & Forster, 2011). Combined with the augmented ventral hippocampus corticosterone response seen in the current study, this suggests a more prominent effect of mineralocorticoid receptor occupancy in the ventral hippocampus during amphetamine withdrawal. Mineralocorticoid receptors in the hippocampus are thought to mediate the effects of corticosterone in increasing anxiety and fear states (Korte, 2001), thus pointing to a central role of enhanced hippocampal corticosterone and more prominent mineralocorticoid receptor

activation in promoting anxiety states during amphetamine withdrawal and representing an important direction to explore in future studies.

### *3.2. Mechanisms mediating enhanced corticosterone in the ventral hippocampus during amphetamine withdrawal*

In the present study, plasma corticosterone levels increased to above stress-naïve levels immediately following 20 minutes of restraint stress, then decreased to below stress-naïve levels 1 hour after restraint. The latter observation is suggestive of negative feedback regulating plasma corticosterone levels post-stress (van Haarst *et al.*, 1997; Russig *et al.*, 2006; Groeneweg *et al.*, 2011; Garrido *et al.*, 2012; Ergang *et al.*, 2014; Laryea *et al.*, 2014). However, amphetamine withdrawal did not alter total, free, or bound levels of either basal (stress-naïve) or stress-induced plasma corticosterone levels relative to those observed in saline-treated controls, suggesting that the capacity for free corticosterone to reach the brain was similar between saline and amphetamine pretreated rats. This lack of difference in stress-induced corticosterone response following 2 weeks of withdrawal from repeated amphetamine is consistent with previous findings using different treatment regimens and/or withdrawal periods. Specifically, Russig *et al.* (2006) showed that escalating doses of amphetamine (1 to 10 mg/kg) given over 4 days did not alter corticosterone either basally or in response to 30 minutes of restraint applied after 2 weeks of withdrawal relative to control levels. Overall, this suggests that amphetamine withdrawal does not alter peripheral corticosterone responses to stress regardless of dose given or length of withdrawal. Further studies are required to determine if this is maintained in response to different types of stress, or is specific to restraint.

With regards to saline pretreated rats, the increase in free corticosterone levels in the plasma was not mirrored by a significant increase in stress-induced ventral hippocampal corticosterone. Similar findings have been observed by others (Droste *et al.*, 2009a; Droste *et al.*, 2009b; Garrido *et al.*, 2012), supporting the suggestion that “a containment mechanism” may exist that prevents overexposure of the brain to glucocorticoid hormones (Droste *et al.*, 2009a). Corticosterone binding globulin (CBG), 11 $\beta$ -HSD1, and the multidrug resistance I (MDR1) type P glycoprotein in the blood-brain barrier have been proposed as three likely containment mechanisms (de Kloet *et al.*, 2005; Droste *et al.*, 2009a; Garrido *et al.*, 2012), that could result in stress-induced increases of corticosterone in the plasma but not in the central nervous system.

Interestingly, the ratio of free to bound corticosterone was found to be approximately 1:3 in both control and withdrawal conditions in the current study whereas previous findings suggest this ratio is typically 1:10 for rodents (Moisan *et al.*, 2014; Sivukhina & Jirikowski, 2014). Typically, approximately 95% of serum corticosteroids are bound by the binding proteins albumin or CBG, which bind 5-15% and 80-85% of circulating corticosteroids respectively (Westphal, 1971; Brien, 1981; Pugeat *et al.*, 1981; Sugio *et al.*, 1999; Baker, 2002; Breuner & Orchinik, 2002; Moisan *et al.*, 2014; Sivukhina & Jirikowski, 2014). One explanation for the current discrepancy is 2 weeks of daily intraperitoneal injections may have altered ratio of free to bound corticosterone. In support of this, a single intraperitoneal injection can elicit a corticosterone stress response in the plasma (Meijer *et al.*, 2006) and stress exposure can reduce plasma CBG levels, contributing to greater prevalence of free unbound corticosterone (Fleshner *et al.*, 1995; Spencer *et al.*, 1996). Thus, it seems plausible that in the present study, 2 weeks of daily intraperitoneal injections may reduce CBG expression or binding in the plasma during the

second week of withdrawal, resulting in an altered ratio of bound to free corticosterone content. This possibility should be investigated in the future.

The current findings did not demonstrate an effect of either amphetamine withdrawal or restraint stress on the expression or activity of  $11\beta$ -HSD1, or on the expression of H6PD, in the ventral hippocampus. The lack of difference in stress-evoked  $11\beta$ -HSD1 expression or activity is in contrast to the finding that exposure to chronic or repeated stress results in increased  $11\beta$ -HSD1 mRNA expression in the CA1 region of the ventral hippocampus (Ergang *et al.*, 2014; Vodicka *et al.*, 2014). Combined, these results suggest that unlike repeated stress, acute stress does not increase  $11\beta$ -HSD1 and thus the capacity to synthesize corticosterone in the ventral hippocampus. However, the effects of a single stressor on these end measures may be expressed at time points beyond the 1 hour post-stress time point used here. Overall, it appears that increased stress-induced corticosterone in the ventral hippocampus of rats undergoing amphetamine withdrawal cannot be explained by greater peripheral synthesis or release of corticosterone, or by enhanced free circulating corticosterone, nor by increased capacity for local synthesis of corticosterone in the ventral hippocampus.

One possible explanation for increased stress-induced corticosterone in the ventral hippocampus shown by amphetamine-withdrawn rats is that amphetamine exposure and withdrawal causes damage to the blood brain barrier that enables the passage of bound corticosterone into the central nervous system, ultimately resulting in greater free levels (as measured by microdialysis) upon dissociation from the binding protein. In support of this possibility, methamphetamine exposure and withdrawal has been reported to increase the permeability of the blood brain barrier to otherwise impermeable binding proteins (Sharma & Ali, 2006; Kiyatkin & Sharma, 2012). Furthermore, failure to show differences in bound steroid

in the plasma, as in the current study, does not preclude increased binding protein in the cerebrospinal fluid (Schwarz & Pohl, 1994). It is therefore conceivable that chronic amphetamine exposure and withdrawal increases the permeability of the blood brain barrier to binding proteins such as CBG, which is known to play a critical role in maintaining a corticosterone pool accessible to central tissues following stress (Mattos *et al.*, 2013). Greater amounts of bound corticosterone entering through a more permeable blood brain barrier would then be available to the ventral hippocampus, where corticosterone could dissociate from CBG to become biologically active. This is in accordance with the higher levels of corticosterone in dialysates collected from amphetamine-withdrawn rats, which represent unbound (active) corticosterone levels.

Alternatively, it is also plausible that amphetamine exposure decreases expression or binding capacity of CBG in cerebrospinal fluid or central tissues such as the ventral hippocampus, resulting in greater free levels of stress-evoked corticosterone in this region. Expression of both CBG mRNA and protein have been identified that are localized to the hippocampus (Sivukhina *et al.*, 2006; Sivukhina *et al.*, 2013a; Sivukhina *et al.*, 2013b), and it appears that CBG expression in hippocampal tissue is intrinsic rather than derived from cerebrospinal fluid or plasma (Orchinik *et al.*, 1997; Jirikowski *et al.*, 2007; Sivukhina & Jirikowski, 2014). Thus, it is possible that decreased extracellular CBG could prolong stress-induced elevations in free extracellular corticosterone levels in the ventral hippocampus that are not observed in the plasma.

Finally, although no effect of amphetamine withdrawal or restraint stress was observed on 11 $\beta$ -HSD1 or H6PD expression or activity in ventral hippocampus tissue, it is possible that amphetamine exposure and withdrawal alters these measures in the cerebrospinal fluid-secreting



cells of the choroid plexus epithelium to cause changes in regulation of corticosterone metabolism in the cerebrospinal fluid. In support of this, 11 $\beta$ -HSD1 and H6PD mRNA expression have been identified in human and rabbit choroid plexus epithelial cells, suggesting the potential to regulate central glucocorticoid availability (Sinclair *et al.*, 2007; Sinclair *et al.*, 2010). Therefore, alterations in 11 $\beta$ -HSD1 or H6PD expression or activity in the choroid plexus of amphetamine-withdrawn rats may represent a mechanism contributing to enhanced stress-induced corticosterone levels in the ventral hippocampus but not in the plasma.

### 3.3. Conclusions

Our findings demonstrate for the first time that withdrawal from chronic amphetamine exposure results in enhanced levels of stress-induced corticosterone in the ventral hippocampus, which may contribute to the related increases in anxiety and sensitivity to stress (Vuong *et al.*, 2010; Li *et al.*, 2014; Tu *et al.*, 2014). We show further that enhancement in stress-induced hippocampal corticosterone is not mediated by either peripheral changes in plasma corticosterone levels or by functional expression of enzymes in the ventral hippocampus that regulate local corticosterone availability. Future work could explore the role of corticosteroid binding globulin in the delivery and regulation of hippocampal corticosterone during amphetamine withdrawal. Furthermore, identifying whether enhanced corticosterone in the ventral hippocampus contributes to enhanced sensitivity to stress during withdrawal may identify the glucocorticoid system as a target for treating amphetamine withdrawal.

## 4. Experimental Procedures

### *4.1 Rodent model of amphetamine pretreatment and withdrawal*

Procedures for all experiments in this study were approved by the Institutional Animal Care and Use Committee of the University of South Dakota, and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For all experiments, male Sprague-Dawley rats (Animal Resources Center, University of South Dakota) were weaned at 3 weeks of age and housed in pairs at a constant room temperature of 22° C (60% relative humidity) with a reverse 12-hour light/dark cycle, (lights off at 10:00 a.m.) and freely accessible food and water. At 8 weeks of age, cage-paired rats were randomly assigned to receive either saline or *d*-amphetamine injections (2.5 mg/kg, ip.) for 14 consecutive days (Barr *et al.*, 2010; Vuong *et al.*, 2010; Barr & Forster, 2011; Li *et al.*, 2014; Tu *et al.*, 2014). Injections were administered during the dark phase of the reverse photoperiod (between 11:00 a.m. and 2:00 p.m.). Following this, rats underwent a 2-week withdrawal period, a protocol that reliably enhances anxiety-like behaviors (Vuong *et al.*, 2010; Reinbold *et al.*, 2014; Tu *et al.*, 2014). This same amphetamine treatment and withdrawal regime has been previously shown to produce heightened anxiety-like behaviors (Barr *et al.*, 2010; Vuong *et al.*, 2010; Reinbold *et al.*, 2014; Tu *et al.*, 2014), stress-induced behavioral arousal (Li *et al.*, 2014), and blunted stress-induced serotonin release in the ventral hippocampus (Li *et al.*, 2014).

### *4.2 Experiment 1 – Amphetamine withdrawal enhances stress-induced corticosterone levels in the ventral hippocampus*

The purpose of this experiment was to determine the effects of amphetamine withdrawal on restraint stress-induced corticosterone in the ventral hippocampus.

#### *4.2.1 Surgical procedures*

During the first week of withdrawal from amphetamine, 13 rats underwent aseptic stereotaxic surgery under isoflurane anesthesia (3% isoflurane: 0.3 mL/min oxygen) (Li *et al.*, 2014). Following anesthesia, rats were placed into a stereotaxic frame and implanted unilaterally with a guide cannula (21 gauge; Plastics One, Roanoke, VA, USA) 2 mm above the ventral hippocampus. Guide cannula coordinates relative to bregma were AP -5.2 mm, ML  $\pm$ 4.4 mm and DV -8.3 mm (Paxinos & Watson, 1998). The pedestal was anchored to the skull with a combination of support screws, glass ionomer cement (Fuji Plus dental acrylic; Patterson Dental, Minneapolis MN, USA) and cranioplastic acrylic (Plastics One). Following surgery, rats were treated with an analgesic (Ketoprofen, 5mg/kg, im.; Med-Vet, Libertyville, IL, USA) and allowed to recover for 3 days (Li *et al.*, 2014) before undergoing further experimental procedures.

#### *4.2.2 Microdialysis*

Approximately 18 hours prior to experimentation, rats were anesthetized with isoflurane and implanted with a laboratory constructed microdialysis probe (Li *et al.*, 2014) that projected 8.3 mm below the cortical surface and had a membrane length of 3.0 mm (MW cut-off 5-6 kDa; Farmer et al., 1996). Free corticosterone (0.35 kDa) can readily pass through the membrane, but bound corticosterone is excluded since the corticosterone binding globulin is 50-60kDa.

The probes were attached to a liquid swivel (Instech Laboratories, Plymouth Meeting, PA, USA) that allowed for free movement of the rat within a 10-gallon aquarium. Artificial cerebrospinal fluid (4.295 g NaCl, 0.1005 g KCl, 0.062g NaH<sub>2</sub>PO<sub>4</sub>, 0.0995 g Na<sub>2</sub>HPO<sub>4</sub>, 0.1015 g

MgCl, and 0.088 g CaCl<sub>2</sub> in 500mL distilled water; pH 7.2; Sigma-Aldrich, St Louis, MO, USA) was perfused through the probe overnight at a flow rate of 0.5 µL/minute.

Flow rate was increased to 0.7 µL/minute prior to sample collection, with collection of baseline samples initiated one hour after the start of the dark phase of the reverse photoperiod and continuing at 20 minute intervals. Once 3 baseline samples had been collected, rats were subjected to restraint stress for 20 minutes. Restraint was carried out as previously reported (Mo *et al.*, 2008; Lukkes *et al.*, 2009; Li *et al.*, 2014) by using polyvinyl chloride tubes (27.3 cm x 5.1 cm x 5.1 cm) (Stamper *et al.*, 2015) that prevent movement while still enabling respiration, thus imposing a psychological but not physical stressor. Following the 20 minute restraint, dialysate samples were collected for a further 120 minutes.

#### 4.2.3 Histology

Following final collection of dialysates, rats were killed by an overdose of sodium pentobarbital (0.5 mL Fatal Plus, ip.; Vortech, Dearborn, MI, USA). Brains were removed and fixed in a 10% formalin solution (Fisher Scientific, Pittsburgh, PA, USA) for 3 days before sectioning (60 µm) on a sliding microtome. Coronal sections were then examined under a light microscope by two experimenters blind to outcome to confirm correct probe placement. Only rats with probes located to the ventral hippocampus were included in the subsequent analyses.

#### 4.2.4 Hippocampal corticosterone measurement

Measurement of hippocampal corticosterone from dialysates was performed using an enzyme-linked immunoassay kit (Enzo Life Sciences, Farmingdale, NY). Briefly, dialysates (7.3 µL) were diluted with 102.7 µL of assay buffer for a 15-fold dilution. Duplicates of each sample were

treated with 0.5  $\mu$ L of SDR, and duplicates of each sample, standard, and control were then assayed. Corticosterone levels were detected using an automatic plate reader (Bio-Tek Instruments, Winooski, VT, USA) at an absorbance of 405 nm, with wavelength correction set at 595 nm. Samples were compared to known standards and absorbance values were used to calculate maximum binding percent (14.2-19% range) and percent of non-specific binding (1.9-2.9%). The detection limit sensitivity of the assay was 27.0 pg/mL. Dialysate levels were not corrected for differences in probe recovery. To account for this, dialysate levels were expressed as the % of the average of the baseline samples (Keeney *et al.*, 2006; Droste *et al.*, 2008; Droste *et al.*, 2009a; Dorey *et al.*, 2012; Barr *et al.*, 2013; Li *et al.*, 2014).

#### *4.3. Experiment 2 – Mechanisms mediating enhanced corticosterone in the ventral hippocampus during amphetamine withdrawal*

The purpose of this experiment was to identify mechanisms underlying the enhanced stress-induced corticosterone levels observed in the ventral hippocampus during amphetamine withdrawal in Experiment 1. Specifically, we investigated whether this enhancement was a result of either augmented increases in stress-induced plasma corticosterone or elevated expression or activity of ventral hippocampus enzymes responsible for regulating local corticosterone metabolism. The steroidogenic enzymes 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1) and hexose 6 phosphate dehydrogenase (H6PD) were selected for testing based on literature suggesting these enzymes as the most likely candidates to affect corticosterone metabolism in the ventral hippocampus (Seckl, 1997; Harris *et al.*, 2001; Taves *et al.*, 2011; Chapman *et al.*, 2013; Ergang *et al.*, 2014; Vodicka *et al.*, 2014). In contrast to earlier studies measuring mRNA expression of these enzymes (Ergang *et al.*, 2014; Vodicka *et al.*, 2014), we

chose to use western immunoblotting to determine protein expression, since mRNA expression cannot account for either functional protein expression or post-translational modifications. The latter is important, since earlier studies suggest that 11 $\beta$ -HSD1 can be glycosylated to alter enzyme activity, and glycosylation may stabilize 11 $\beta$ -HSD1 reductase function (Seckl, 1997). Therefore, in addition to measuring protein levels of 11 $\beta$ -HSD1 and H6PD, we also conducted an enzyme activity assay to test the hypothesis that amphetamine withdrawal is associated with increased activity of 11 $\beta$ -HSD1 in the ventral hippocampus, which may contribute locally to enhanced stress-induced corticosterone in that region during amphetamine withdrawal.

#### *4.3.1 Physical restraint*

Restraint stress (as described for Experiment 1) was applied for 20 minutes to a separate group of amphetamine or saline pretreated rats (n=70 total, 11-12 per treatment group) during the second week of withdrawal. Following restraint, rats from each pretreatment group were either decapitated immediately (n=11-12/group), or were transferred back to the home cage for one hour where they remained until decapitation (n=11-12/group). Stress-naïve controls (n=12/group) were also sampled to provide baseline measures. All sampling (from 20 minute restraint, 20 minute restraint + 1 hour recovery, and stress-naïve rats) occurred between 11:00 a.m. and 1:00 p.m., with collection from stress-naïve controls time-matched to the stress-groups.

#### *4.3.2 Blood and tissue collection*

To eliminate the confound of anesthesia, rats were rapidly decapitated following stress or control conditions. Trunk blood was collected in heparinized tubes and brains were rapidly removed and frozen on dry ice after collection. Blood samples were centrifuged at 5000 rpm, with plasma

then drawn off. Both plasma and brain tissue were stored at -80 °C until processing.

#### *4.3.3 Plasma corticosterone measurement*

Corticosteroid binding globulin (CBG) has been implicated in regulating the fast actions of glucocorticoids on central tissues (Moisan *et al.*, 2014) and in contributing to neuroendocrine stress response phenotypes in mice (Richard *et al.*, 2010; Mattos *et al.*, 2013), with chronic exposure to morphine increasing CBG in rats (Nock *et al.*, 1997). To test whether amphetamine withdrawal alters plasma corticosterone levels or corticosterone binding to CBG, plasma levels of bound, unbound, and total corticosterone were quantified using an enzyme immunoassay kit (as described for ventral hippocampal samples in Experiment 1), with the following modifications: 10 µL duplicate samples of plasma from each subject were diluted (1:100) with 990 µL assay buffer, and all samples were assayed in duplicate, but one of each pair was treated with 0.5 µL of SDR. This enabled measurement of total corticosterone levels from the non SDR-treated sample, while the SDR-treated sample provided levels of unbound corticosterone. Unbound levels were then subtracted from total levels to provide a measure of bound corticosterone. All corticosterone measurements were expressed as ng/mL of plasma. The average percentages of maximum and non-specific binding were 18.3% and 2.5% respectively.

#### *4.3.4 Western immunoblotting*

Brain tissue was sliced into 300 µm coronal sections in a cryostat maintained at -10 °C. The ventral hippocampus was located according to the rat brain atlas of Paxinos and Watson (1998) and microdissected on a freezing stage (Physitemp; North Central Instruments, Inc., Plymouth, MN, USA) using a 20 gauge cannula (Barr & Forster, 2011; Barr *et al.*, 2013). Tissue samples

were then expelled into 60  $\mu$ L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing protease inhibitor (28  $\mu$ L/mL HEPES) and homogenized by sonication. Protein concentrations of each sample were determined by Bradford immunoassay (Bradford, 1976) with remaining samples stored at -80 °C until processed. Equal amounts of each sample (40  $\mu$ g of protein per lane) were loaded onto 10% SDS-polyacrylamide gels for western immunoblotting. The blotted membranes were then blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) and 5% non-fat dry milk (NFDM) for 1 hour at room temperature prior to incubation for 18 hours at 4 °C in TBST with primary antibodies (rabbit host) against either 11 $\beta$ -HSD1 (Cat #sc-20175, 1:200, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or-H6PD (Cat #sc-67394, 1:1,000 Santa Cruz). Membranes were washed three times with TBST at room temperature, and then incubated for 2 hours at room temperature with IRDye800-conjugated polyclonal goat anti-rabbit IgG secondary antibody (Cat #611-132-002, 1:1000, Rockland Inc., Gilbertsville, PA, USA). After this, membranes were washed three times with TBST before visualization. Primary antibodies for 11 $\beta$ -HSD1 and H6PD were selected based on published literature (Xu *et al.*, 2012; Uschold-Schmidt *et al.*, 2013; Kovacevic *et al.*, 2014; Vasiljevic *et al.*, 2014). To control for protein loading, each membrane was also incubated in mouse anti-actin clone C4 primary antibody (Cat #MAB1501R, 1:2,000, EMD Millipore Corp., Darmstadt, Germany) and IRDye 800-conjugated anti-mouse secondary antibody (Cat #610-132-003, 1:5000, Rockland Inc.). Membranes were scanned in a LI-COR scanning machine with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) using an 800 nm range filter to visualize proteins, as described previously (Barr & Forster, 2011). Optical densities of the protein bands for 11 $\beta$ -HSD1 or H6PD for each individual sample were expressed as a mean percentage of the optical density for the  $\beta$ -actin control in each treatment condition.



Solutions were made in our laboratory with reagents purchased from Sigma-Aldrich (St. Louis, MO, USA) with the exception of Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics Corp., Indianapolis, IN, USA).

#### *4.3.5 Ex vivo enzyme activity assay*

The 11 $\beta$ -HSD1 activity assay employed here is based on previously published reports in frozen *ex vivo* hippocampal tissue (Low *et al.*, 1994; Jellinck *et al.*, 1997; Sooy *et al.*, 2010). Ventral hippocampal tissue remaining from the western immunoblotting assay was microdissected from frozen sections and expelled into 60  $\mu$ L of Krebs-Ringer buffer (Low *et al.*, 1994) and sonicated (Fisher Scientific Model FB50 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA). Protein concentrations of each sample were determined using Bradford immunoassay (Bradford, 1976), with remaining samples stored at -80 °C until processing. Tissue samples were then diluted with Krebs-Ringer buffer to 0.5 mg/mL (Low *et al.*, 1994; Sooy *et al.*, 2010), and 70  $\mu$ L aliquots of diluted sample from each subject were incubated for 1 hour at 37°C (Sooy *et al.*, 2010) with 70  $\mu$ L of active or control buffer C: 1mM EDTA, 300 mM NaCl, 100 mM potassium acetate, 10% glycerol, 10 mM D-glucose-6-phosphate dipotassium salt hydrate, pH 6.0, with “active” buffer C containing 10 nM 11-Dehydrocorticosterone (DHC) substrate and “control” buffer C containing no 11-DHC substrate (Brown *et al.*, 1993; Sooy *et al.*, 2010). Both reaction conditions were run in duplicate for each sample and all reactions were stopped with 140  $\mu$ L ice-cold methanol and stored at -80 °C (Senesi *et al.*, 2010). Samples were then centrifuged (Senesi *et al.*, 2010) at 14,000 rpm at 4 °C for 11 minutes, and the supernatant drawn off and assessed for corticosterone levels (ng/mL) using a corticosterone enzyme immunoassay kit (Enzo Life Sciences) as described in Section 4.3.3, with the following modifications: supernatant was

treated with 0.5  $\mu$ L SDR, diluted (1:10) in corticosterone assay buffer and neutralized (pH 7.0). Samples for each subject were run in duplicate, and corticosterone levels in “active” buffer samples were expressed as percentage of “control” buffer samples to provide indirect measures of 11 $\beta$ -HSD1 activity. The average maximum binding was 17.8% and the average nonspecific binding was 2.8%. Levels of corticosterone in the samples were within the detection limit of the assay (27 pg/mL). All reagents were purchased from Sigma-Aldrich, except 11-dehydrocorticosterone (United States Biological, Swampscott, MA, USA).

#### 4.4 Data analysis

Analyses were performed using SigmaStat v3.5 (SPSS Inc., Point Richmond, CA) with a p-value of  $P < 0.05$  considered significant. A total of eight data points across all analyses were excluded using the Grubbs’ outlier test (Grubbs, 1969). Baseline extracellular corticosterone concentrations (uncorrected for probe recovery) were compared between saline and amphetamine pretreated rats using a paired t-test. Hippocampal corticosterone (% of pre-stress levels) was then analyzed among treatment with two-way mixed design ANOVA with one repeated measure (time). One-way repeated ANOVA was used to detect a significant main effect of time within saline-pretreated or amphetamine-pretreated rats, with Holm-Sidak *post-hoc* tests for multiple comparisons against a baseline (-20 minutes) used to identify which time points differed. A significant interaction between treatment and time was followed up with Student-Newman-Keuls (SNK) *post-hoc* tests for multiple comparisons to identify which time points differed between treatments. Plasma corticosterone, hippocampal protein levels of 11 $\beta$ -HSD1 and H6PD, and corticosterone levels from the hippocampal 11 $\beta$ -HSD1 activity experiment were analyzed by 2-way ANOVA (amphetamine / saline pretreatment x stress condition, i.e., restraint

only, restraint + 1 hour recovery, stress-naive), with SNK post-*hoc* tests for multiple comparisons used to follow up main effects.

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## **Figure Legends:**

### **Figure 2-1**

(A) Representative diagrams of microdialysis probe membrane placements in the ventral hippocampus of amphetamine and saline treatment groups. Figure adapted from Paxinos and Watson (1998) bregma -5.20 mm. (B) Restraint stress-induced corticosterone levels in the ventral hippocampus were increased in rats undergoing amphetamine withdrawal. Restraint stress was applied for 20 minutes during the sample period marked by the horizontal bar. N = 7-9 per group. \* significant differences between amphetamine and saline treatment groups; # significantly different from baseline (-20 minute) levels; P < 0.05.

### **Figure 2-2**

(A) Total, (B) free, and (C) bound plasma corticosterone levels increase to above stress-naïve control levels immediately following 20 minutes of restraint stress, then decrease to below stress-naïve control levels after 1 hour, with no significant difference observed between saline and amphetamine pretreated groups (n=9-12 per treatment group per time point, mean ± SEM). #significant difference compared to stress-naïve control group (P < 0.05). \* significant difference compared to restraint (no recovery) group (P < 0.001).

### **Figure 2-3**

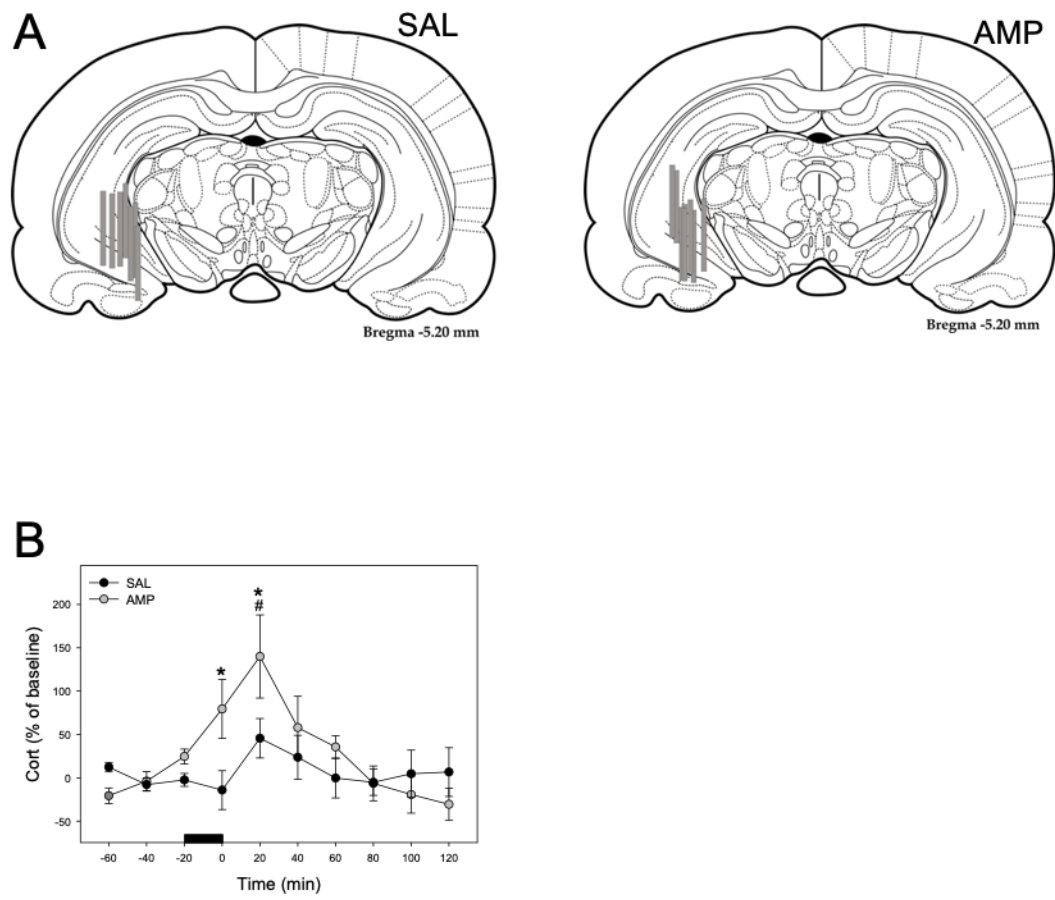
Amphetamine withdrawal does not alter expression of (A) 11β-HSD1 or (B) H6PD in the ventral hippocampus before (No Restraint, n=11), immediately (No Recovery, n=10), or 1 hour (1h Recovery, n=10) after restraint stress, compared to saline controls (11β-HSD1 p=0.977, H6PD p=0.237). Restraint stress did not significantly alter 11β-HSD1 or H6PD expression in the

ventral hippocampus, compared to controls (11 $\beta$ -HSD1  $p=0.741$ , H6PD  $p=0.118$ ). Protein band density values are expressed as mean percent of  $\beta$ -Actin controls  $\pm$  SEM.

#### **Figure 2-4**

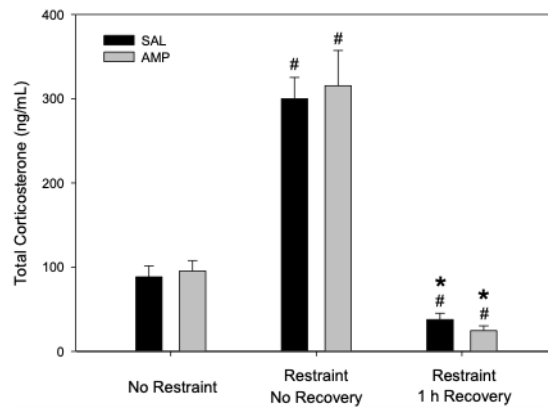
Amphetamine pretreatment (gray bars) does not alter 11 $\beta$ -HSD1 activity in the ventral hippocampus immediately or 1 hour after restraint stress (or in stress-naïve controls) relative to saline pretreatment (black bars;  $n=8-10$  per treatment group, mean  $\pm$  SEM).

**Figure 2-1**

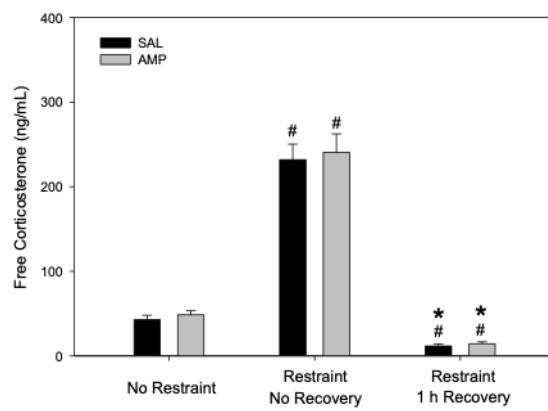


**Figure 2-2**

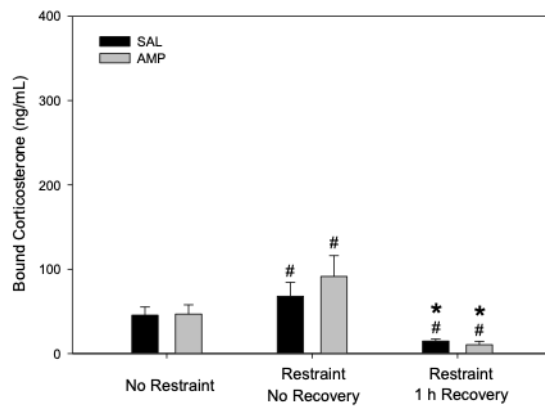
**A**



**B**

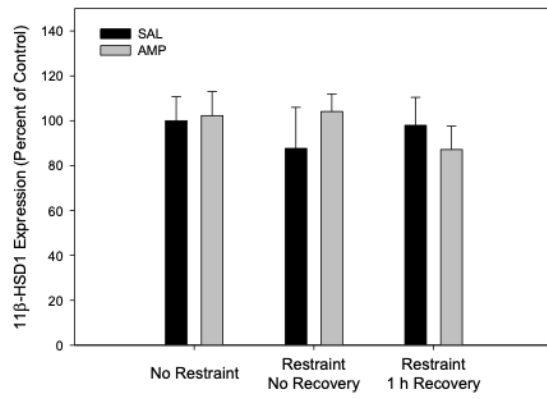


**C**

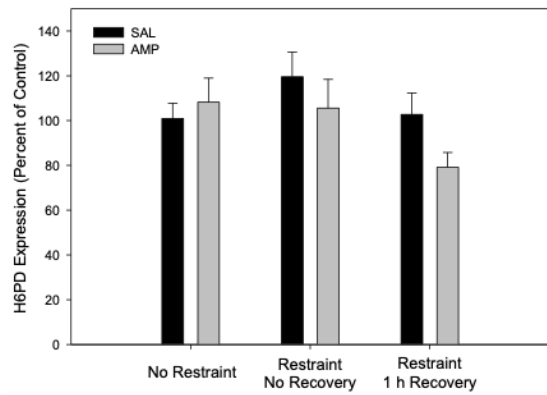


**Figure 2-3**

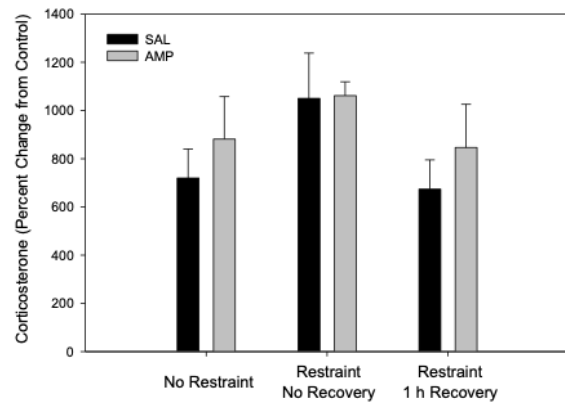
**A**



**B**



**Figure 2-4**





### **Chapter 3: Corticosterone in the ventral hippocampus differentially alters accumbal dopamine output in drug-naïve and amphetamine-withdrawn rats**

#### **Abstract:**

Dysregulation in glucocorticoid stress and accumbal dopamine reward systems can alter reward salience to increase motivational drive in control conditions and contribute to relapse during drug withdrawal. Amphetamine withdrawal is associated with dysphoria and stress hypersensitivity (humans and rodents) that may be mediated by enhanced stress-induced corticosterone in the ventral hippocampus (vHipp) and dysregulated vHipp glucocorticoid receptor signaling (found in rats). vHipp corticosterone is thought to be excitatory and vHipp excitation enhances accumbal shell dopamine release. To test whether vHipp corticosterone can directly alter accumbal dopamine output in control conditions and in amphetamine withdrawal, stress-relevant concentrations of corticosterone or vehicle were infused into the vHipp of urethane-anesthetized adult male rats in control and withdrawal conditions. Accumbal dopamine output was assessed with *in vivo* chronoamperometry. vHipp corticosterone rapidly enhanced accumbal dopamine output in control conditions but produced a biphasic reduction in accumbal dopamine output in amphetamine withdrawal. Selectively blocking glucocorticoid-, mineralocorticoid-, or cytosolic receptors blocked the effects in both conditions. Therefore, we conclude that corticosterone's ability to alter accumbal dopamine output requires cooperative activation of membrane mineralocorticoid receptors and cytosolic glucocorticoid receptors that respectively excite and

disinhibit glutamatergic efferents to the mesoaccumbal system. Enhanced synaptic inhibition within the hippocampal-mesolimbic system may result in excitatory output from the vHipp having an inhibitory effect on accumbal dopamine release in protracted psychostimulant withdrawal. These findings suggest vHipp corticosterone plays an important role in driving positive stress coping mechanisms in healthy conditions and dysregulation of this system may contribute to relapse during withdrawal.

## **1. Introduction**

Stress plays an important role in motivating goal-oriented behavior, which may be through its mediation of dopamine reward systems centrally. For example, stress increases dopamine levels in the nucleus accumbens shell (Kalivas & Duffy, 1995; Enrico *et al.*, 2013), which can enhance motivational drive and direct goal-oriented behavior (Floresco, 2014; Hollon *et al.*, 2015; Berridge & Robinson, 2016). However, stress plays a very different role in psychostimulant withdrawal, where it can *reduce* accumbal shell dopamine levels and prompt negative affect and dysphoria that drive drug-taking behaviors and predict relapse (Sinha, 2007; Cleck & Blendy, 2008; Paliwal *et al.*, 2008; Wheeler *et al.*, 2008; Koob *et al.*, 2014; Twining *et al.*, 2014; Kwako & Koob, 2017).

Amphetamine dependence is a global health problem with high relapse potential and no effective interventions (Gossop, 2009; Sun *et al.*, 2014). Amphetamine withdrawal is characterized by enhanced physiological and behavioral responses to stress (Li *et al.*, 2014; Bray *et al.*, 2016) as well as craving, anxiety, and dysphoria in humans (Gossop, 2009; Shoptaw *et al.*, 2009; Kosten, 2012) and rodents (Cryan *et al.*, 2003; Russig *et al.*, 2006; Li *et al.*, 2014; Tu *et al.*, 2014; Bray *et*

*al.*, 2016). The negative affect states can induce relapse and thus maintain the addiction cycle (Paliwal *et al.*, 2008; Gossop, 2009; Koob *et al.*, 2014). These symptoms may be mediated by alterations in dopamine reward- and corticosterone stress responses (Fig. 1-1) (Koob & Volkow, 2010; Koob *et al.*, 2014; Bray *et al.*, 2016; Barr *et al.*, 2017). For example, stress increases free extracellular corticosterone levels in the ventral hippocampus in rodents (Droste *et al.*, 2008; Droste *et al.*, 2009; Bray *et al.*, 2016) which is enhanced in amphetamine withdrawal (Bray *et al.*, 2016). The ventral hippocampus conveys interoceptive information to the nucleus accumbens shell (Strange *et al.*, 2014; Barr *et al.*, 2017). Accumbal shell dopamine responds differently to stress during drug withdrawal, and can directly drive negative affect, drug-taking, and relapse (Sinha, 2007; Roitman *et al.*, 2008; Twining *et al.*, 2014; Hurley *et al.*, 2017; Haake *et al.*, 2018). Together, these findings suggest that the ventral hippocampal corticosterone stress response (Bray *et al.*, 2016) may be able to mediate the ability of stress exposure to induce craving and dysphoria and drive relapse during drug withdrawal. However, whether corticosterone in the ventral hippocampus *can* alter accumbal dopamine output in psychostimulant withdrawal remains unknown.

Corticosterone is thought to be excitatory in the ventral hippocampus, and can induce glutamate release *in vitro* (Karst *et al.*, 2005; Wang & Wang, 2009). At the cellular level, corticosterone induces its effects primarily by activating mineralocorticoid and glucocorticoid receptors, both of which are highly expressed in the ventral hippocampus (Reul & de Kloet, 1986; Herman *et al.*, 1989). These receptors can be cytosolic or membrane-bound, genomic or non-genomic, and differ in their affinity for corticosterone, downstream signaling mechanisms, and temporal signatures in a regionally-dependent manner (Fig. 1-4) (Groeneweg *et al.*, 2012; Barr *et al.*,

2017; Joels & de Kloet, 2017). In the ventral hippocampus, non-genomic membrane mineralocorticoid receptors can be excitatory or disinhibitory, rapidly enhancing excitatory postsynaptic potential and glutamatergic transmission (Karst *et al.*, 2005; Maggio & Segal, 2007) and reducing inhibitory postsynaptic currents (Maggio & Segal, 2009) *in vitro*. Non-genomic glucocorticoid receptors can be membrane-bound or cytosolic and are thought to act through serotonin- and retrograde signaling mechanisms to enhance or suppress the activity of local (and possibly terminal/extrahippocampal) glutamatergic, GABAergic, and monoaminergic neurotransmission with lock-and-key specificity in a regionally-dependent manner (Fig. 1-4D) (Di *et al.*, 2003; Tasker *et al.*, 2006; Maggio & Segal, 2007; 2009; Wang & Wang, 2009; Hu *et al.*, 2010; Popescu *et al.*, 2010; Barr & Forster, 2011; Groeneweg *et al.*, 2011; Berumen *et al.*, 2012; Chamberland & Topolnik, 2012; Groeneweg *et al.*, 2012; Hill & Tasker, 2012; Di *et al.*, 2016; Nahar *et al.*, 2016; Pehrson *et al.*, 2016; Barr *et al.*, 2017). Overall, corticosterone has rapid effects on local neurotransmission in the ventral hippocampus that have potential to influence activity of limbic structures targeted by this region of the hippocampus.

At the systems level the ventral hippocampus sends glutamatergic projections to the ventral tegmental area and nucleus accumbens shell (Fig. 1-3) and electrical stimulation of the ventral hippocampus or NMDA infusions into this region both independently enhance accumbal shell dopamine output *in vivo* (Blaha *et al.*, 1997; Legault *et al.*, 2000; Floresco *et al.*, 2001; Valenti *et al.*, 2011; Britt *et al.*, 2012; Barr *et al.*, 2014; Taepavarapruk *et al.*, 2014). Thus, it seems plausible that the local effects of corticosterone in the ventral hippocampus could enhance accumbal shell dopamine output in drug naïve conditions, enabling stress to enhance reward salience and motivate goal-oriented behavior. Dysregulation of this system could enable stress to

induce relapse during amphetamine withdrawal. However, whether corticosterone in the ventral hippocampus actually *does* regulate dopamine reward systems has never been directly tested. Here, we directly tested whether a stress-relevant concentration of corticosterone infused into the ventral hippocampus can alter accumbal dopamine output in drug-naïve rats, linking stress to reward salience. We also explored the receptor mechanisms that mediate the ability of corticosterone in the ventral hippocampus to alter accumbal dopamine output, elucidating – for the first time – the specific roles of glucocorticoid- and mineralocorticoid receptors in the cytosol and in the membrane in relation to their modulation of accumbal dopamine release.

Interestingly, repeated amphetamine exposure significantly reduces glucocorticoid receptor expression and glucocorticoid-to-mineralocorticoid receptor ratios in the ventral hippocampus (Barr & Forster, 2011). Glucocorticoid-receptor-mediated serotonin signaling is also impaired in amphetamine withdrawal (Barr & Forster, 2011; Li *et al.*, 2014). This may alter the ability of glucocorticoid receptors to regulate local excitatory/inhibitory tone in the ventral hippocampus (Maggio & Segal, 2009; Barr *et al.*, 2017) since excitatory 5-HT<sub>3</sub> receptors are preferentially located on interneurons and interneuron-specific interneurons (Fig. 1-4; also, see discussion in Appendix 2) (Freund & Buzsaki, 1996; Berumen *et al.*, 2012; Chamberland & Topolnik, 2012; Pelkey *et al.*, 2017). Furthermore, repeated cocaine exposure shifts the excitation/inhibition balance in the nucleus accumbens shell to an excitatory state in acute (< 24 h) withdrawal (Fig. 4-1)(Scudder *et al.*, 2018) and enhances the ability of NMDA infusions in the ventral hippocampus to stimulate accumbal dopamine release (Barr *et al.*, 2014). Therefore, we tested whether repeated amphetamine exposure disrupts the effects of glucocorticoid and

mineralocorticoid receptor activation on accumbal dopamine output in protracted amphetamine withdrawal.

## **2. Materials and Methods**

### *2.1. Animals*

All experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the & Use of Laboratory, 2011) and were approved by the Institutional Animal Care and Use Committee of the University of South Dakota. All efforts were made to minimize animal suffering and reduce the number of subjects used.

A total of 127 adult male Sprague-Dawley rats were used in this experiment ( $367 \text{ g} \pm 35 \text{ g}$ ; range: 248 - 454 g). Rats were obtained from the University of South Dakota Animal Resource Center at 3 weeks of age and pair-housed in polysulfone cages (Tecniplast, Buguggiate, Varese, Italy; 16.73 x 10.47 x 7.28 in, floor area: 800 cm<sup>2</sup>/124 in<sup>2</sup>; corn husk bedding) held at a constant room temperature of 22° C (60% relative humidity) on a reverse 12-h light/dark cycle with lights off from 10:00 – 22:00. To eliminate a possible confound of enrichment-induced hippocampal neurogenesis and enrichment-induced alterations of stress responsiveness (Tanti *et al.*, 2012; Levone *et al.*, 2015), cages contained no enrichment but *ad lib* access to water and standard rat chow.

## 2.2. Rat model of amphetamine pretreatment and withdrawal

At 8 – 10 weeks of age (average age: postnatal day [PND]  $57 \pm 3.5$  days; range: 46 – 66 days), cage-paired rats were randomly assigned to receive daily injections of physiological saline ( $n = 68$ ) or d-amphetamine sulfate ( $n = 44$ ) (2.5 mg/kg b.w., i.p.) for 14 days (Barr & Forster, 2011; Li *et al.*, 2014; Bray *et al.*, 2016) followed by a 14-day withdrawal period (Li *et al.*, 2014; Tu *et al.*, 2014; Bray *et al.*, 2016; Solanki *et al.*, 2016) (except for 3.5, when only saline injections were administered). d-amphetamine sulfate (SigmaAldrich) was dissolved in physiological saline (0.9%) and administered i.p. (2.5 mg/kg b.w.), with an equivalent volume of physiological saline (0.9%) administered to controls (Barr *et al.*, 2010; Vuong *et al.*, 2010; Barr & Forster, 2011; Li *et al.*, 2014; Tu *et al.*, 2014; Bray *et al.*, 2016). To control for diurnal corticosterone levels (van Haarst *et al.*, 1997; Tye *et al.*, 2009), all injections were administered during the dark phase of the reverse photoperiod, between 11:00 and 14:00. This amphetamine protocol has been shown to enhance stress responses, including anxiety (Vuong *et al.*, 2010; Reinbold *et al.*, 2014; Tu *et al.*, 2014), stress-induced behavioral arousal (Li *et al.*, 2014), and corticosterone stress responses (and abolish *serotonin* stress responses) in the ventral hippocampus (Li *et al.*, 2014; Bray *et al.*, 2016).

## 2.3. Stereotactic surgery

After the second week of withdrawal from amphetamine or saline pre-treatment (at PND  $87 \pm 4$  days; range: 77 – 98 days), rats were anesthetized with urethane (0.345 g/mL, 1.8 g/kg, i.p.). Urethane is a long-acting anesthetic that does not affect endogenous dopamine clearance (Blaha *et al.*, 1997; Sabeti *et al.*, 2003; Barr & Forster, 2011; Novick *et al.*, 2015). Upon cessation of pedal withdrawal and eye blink reflexes, rats were placed into a stereotaxic frame (Kopf,

Tujunga, CA, USA) with incisor bar set at -3.5 mm and body temperature held at  $36^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  by a temperature-controlled heating pad (Harvard Apparatus, Holliston, MA, USA). A 22-gauge stainless-steel guide cannula was implanted into the right or left ventral hippocampus (-5.2 mm AP from bregma;  $\pm 4.5$  mm ML; -4.5 mm DV from dura) (Paxinos & Watson, 1998; Tu *et al.*, 2014) (Figs. 3-2B, 3-5C) and a silica infusion cannula (2 mm > guide) was inserted through the guide cannula.

#### 2.4. *In vivo electrochemistry*

A custom-made stearate-treated carbon paste recording electrode with 200  $\mu\text{m}$  recording surface diameter (Blaha & Jung, 1991; Miller *et al.*, 2005; Tye *et al.*, 2009; Novick *et al.*, 2015) was implanted into the ipsilateral medial nucleus accumbens shell (1.6 mm AP from bregma;  $\pm 0.7$  mm ML; -7.0 mm DV from dura) (Paxinos & Watson, 1998; Miller *et al.*, 2005) (Figs. 3-2A, 3-4B, 3-5) to measure dopamine oxidation current without interference from other oxidizing species (Blaha & Lane, 1983; Blaha, 1996; Miller *et al.*, 2005; Tye *et al.*, 2009; Novick *et al.*, 2015) (Fig. 3-1). A custom-made reference electrode with AgCl-coated tip was placed touching the contralateral cortical tissue and a stainless-steel auxiliary electrode was fixed to the skull with a stainless steel surgical screw (Miller *et al.*, 2005; Novick *et al.*, 2015). Prior *in vitro* electrode recordings were conducted to confirm a distinct and measurable dopamine oxidation signal after systemic addition of exogenous dopamine, norepinephrine, and ascorbic acid (Novick *et al.*, 2015; Weber *et al.*, 2018).

Following a thirty-minute recovery period, voltammetry sweeps were conducted using an electrometer (Echempro, GMA Technologies, Inc., Vancouver, Canada) to confirm dopamine



detection and identify the range of potentials to be applied to the electrode during chronoamperometry (Miller *et al.*, 2005; Novick *et al.*, 2015) (Fig. 3-1). This was done by applying an incrementally increasing range of electrical potentials to the working electrode (-150 mV to +450 mV vs. Ag/AgCl, ramp rate 10 mV/second; (Novick *et al.*, 2015)). A 300 mV voltammetric range encompassing the distinct dopamine signal was then selected and the electrometer was set to repetitively apply that range of potential (typically -150 mV to +150 mV vs. Ag/AgCl reference electrode) to the working electrode in brief (1 second) pulses at 30 s intervals, with changes in dopamine oxidation current recorded in ampere at the end of each pulse and converted to nA for analysis (Borland & Michael, 2007; Novick *et al.*, 2015).

After 30 min of stable baseline recordings of the oxidation current, agents were infused into the ventral hippocampus as described below (2.5). Dopamine oxidation current recordings were subsequently run until they returned to basal levels (~2 hours post-infusion). Rats were checked for sedation level throughout the experiment and upon termination of oxidation recordings, rats were euthanized with a lethal dose of FatalPlus (Vortech, Dearborn, MI, USA; 0.5 mL, i.p.) or Euthasol (Virbac, Fort Worth, TX, USA; 0.5 mL, i.p.) followed by decapitation and brain removal for histology.

## 2.5. Microinfusions

All infusions (3.1 – 3.5) were administered through a silica infusion cannula using a microinfusion pump (Stoelting, Wood Dale, IL, USA) at a rate of 0.5  $\mu$ L total volume infused over one minute, with the cannula left in position for the duration of the experiment (Scholl *et al.*, 2010; Weber *et al.*, 2018). Infusions were administered during the dark phase of the reverse

photoperiod, between 13:00 and 18:00. (average infusion time was  $15:30 \pm 1:18$ ). Corticosterone (SigmaAldrich) was dissolved in 2-hydroxypropyl- $\beta$ -cyclodextrin [HBC] (Tocris) (0.05%) then diluted with artificial cerebrospinal fluid (aCSF) to produce a  $0.48\text{ng}/\mu\text{L}$  concentration ( $0.24\text{ ng}$  total delivered, equal to  $6.927 \times 10^{-13}\text{ M}$  of corticosterone); this concentration has been demonstrated previously to be stress-relevant when infused into the ventral hippocampus (Barr & Forster, 2011), thus infusing this amount into the ventral hippocampus here mimics the corticosterone stress response previously observed in the hippocampus (Droste *et al.*, 2008; Bray *et al.*, 2016). Corticosterone alone or vehicle alone (0.05% HBC diluted in aCSF) were infused into the ventral hippocampus of rats in the second week of withdrawal from saline- or amphetamine pre-treatment (saline pre-treatment + corticosterone infusions  $n = 6$ ; saline pre-treatment + HBC infusions  $n = 3$ ; amphetamine pre-treatment + corticosterone infusions  $n = 5$ ; amphetamine pre-treatment + HBC infusions  $n = 4$ ) (3.1 and 3.3). The data for these rats were grouped and analyzed together with that obtained after infusing  $0.5\text{ }\mu\text{L}$  of the vehicle for antagonist infusions in 3.4 (1:1 solution of 5% ethanol and 5% Kolliphor EL) ten minutes prior to infusing corticosterone or HBC into the ventral hippocampus of drug-naïve and amphetamine withdrawn rats (saline pre-treatment + vehicle + corticosterone infusions  $n = 6$ ; saline pre-treatment + vehicle + HBC infusions  $n = 6$ ; amphetamine pre-treatment + vehicle + corticosterone infusions  $n = 6$ ; amphetamine pre-treatment + vehicle + HBC infusions  $n = 5$ ) (Fig. 3-2 and 3-3A).

To uncover the role of glucocorticoid- and mineralocorticoid receptors in mediating the observed effects of ventral hippocampal corticosterone infusions on accumbal dopamine output (3.4), glucocorticoid- and mineralocorticoid receptors were independently blocked by infusing

selective doses of either the glucocorticoid receptor antagonists mifepristone (Barr & Forster, 2011) or the mineralocorticoid receptor antagonist spironolactone (Garthwaite & McMahon, 2004) into the ventral hippocampus ten minutes prior to corticosterone or HBC infusions in drug-naïve and amphetamine-withdrawn rats (mifepristone + corticosterone  $n = 12$  (6 saline- and 6 amphetamine pre-treated); mifepristone + HBC  $n = 13$  (6 saline- and 7 amphetamine pre-treated); spironolactone + corticosterone  $n = 14$  (7 saline- and 7 amphetamine pre-treated); spironolactone + HBC  $n = 14$  (7 saline- and 7 amphetamine pre-treated)) (Figs. 3-2 and 3-3B-C).

Mifepristone and spironolactone (Sigma Aldrich) were infused using concentrations of 2.5 mg/mL in vehicle [1:1 solution of 100% ethanol (Fisher Scientific) and 5% Kolliphor EL (SigmaAldrich), diluted in aCSF]; 1.25  $\mu$ g total delivery for both antagonists; equal to 2.91 nM mifepristone and 2.99 nM spironolactone (or vehicle). Mifepristone was used as a glucocorticoid receptor antagonist with dose selected based on previous use in our laboratory (1.25 ng in 0.5  $\mu$ L) (Barr & Forster, 2011). Mifepristone is also a progesterone receptor antagonist with a higher affinity for progesterone receptors than for glucocorticoid receptors (Heikinheimo & Kekkonen, 1993; Heikinheimo, 1997; Mahajan & London, 1997). Therefore, our dose was selected based on its ability to block glucocorticoid receptors and we must accept that progesterone receptor inhibition also occurs in these infusions. Spironolactone was used as a mineralocorticoid receptor antagonist with dose selected based on specificity for mineralocorticoid receptors (2.99 nM) (Garthwaite & McMahon, 2004).

To determine whether the observed effects of ventral hippocampal corticosterone infusions on accumbal dopamine output (3.1) were mediated by membrane- or cytosolic receptors, 4-pregnen-

11b, 21-diol-3, 20-dione 3-carboxymethyloxime : BSA, (Corticosterone 3-CMO : BSA, Steraloids, Inc.) was used, in which corticosterone is commercially conjugated to bovine serum albumin via carboxymethyloxime-ketone conjugation at corticosterone's terminal ketone group. BSA is hydrophobic and thus its conjugation to corticosterone prevents corticosterone from crossing the plasma membrane to act on cytosolic receptors (Morozov *et al.*, 1988; Groeneweg *et al.*, 2011). The corticosterone 3-CMO : BSA conjugate was dissolved in 0.05% HBC then diluted with aCSF to produce a 5.95 ng/ $\mu$ L concentration (2.97 ng total delivered, for a total of 0.24 ng of corticosterone delivered per 0.5  $\mu$ L infusion). For the BSA vehicle infusions, BSA (SigmaAldrich) was dissolved in 0.05% HBC then diluted in aCSF to produce a 5.4 ng/ $\mu$ L concentration (2.7 ng of BSA total delivered per 0.5  $\mu$ L infusion, equal to the amount of BSA delivered in the 0.5  $\mu$ L infusions of Corticosterone 3-CMO : BSA).

Corticosterone 3-CMO : BSA ( $n = 8$ ) or its vehicle (BSA-HBC,  $n = 7$ ) were infused into the ventral hippocampus of saline pre-treated rats only (Fig. 3-4). Corticosterone 3-CMO : BSA was infused at a concentration of 2.6485 ng / 0.5  $\mu$ L infusion, which contains 0.24 ng of corticosterone / 0.5  $\mu$ L infusion – the same stress-relevant concentration of free/unconjugated corticosterone infused into the ventral hippocampus in 3.1 and the same amount of free/unconjugated corticosterone previously observed in the ventral hippocampus in response to stress *in vivo* (Droste *et al.*, 2008).

## 2.6. Histology

Following euthanasia with 0.5 mL sodium pentobarbital i.p., rat brains were removed and fixed in 10% buffered formalin (Fisher Scientific) for  $\geq 72$  hours, sectioned on a sliding microtome (60

$\mu\text{m}$  at  $-12^{\circ}\text{C}$  maintained with dry ice), and analyzed under a light microscope by two individuals blind to treatment, to confirm cannula and electrode placement and identify anatomical controls (Figs. 3-2, 3-4B-C, 3-5B-C) (Barr & Forster, 2011; Novick *et al.*, 2015).

## 2.7. Data analysis and statistics

All statistical analyses were performed using IBM® SPSS® Statistics v25- (SPSS Inc., Armonk, NY) and SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA) software with alpha level set at 0.05 throughout.

To analyze chronoamperometry results, pre- and post-infusion recordings for each individual subject were separately normalized to zero current values, with changes after microinfusions reported as absolute changes in dopamine oxidation current, as previously published (Novick *et al.*, 2015). Data points were collected every 30 s, but were collapsed into 90 s time bins for repeated measure of time analysis. Two distinct Grubbs outlier tests (Grubbs, 1969) were run at the two non-adjacent points farthest from zero in each treatment group. All data points from subjects whose dopamine oxidation current values were identified to be Grubbs outliers at either of the two non-adjacent points farthest from zero in each treatment group's average datum were excluded as Grubbs outliers, for a total of six rats. These six rats included saline pre-treated rats receiving infusions of: vehicle + corticosterone ( $n = 1$ ), spironolactone + corticosterone ( $n = 1$ ), and BSA-HBC ( $n = 1$ ); and amphetamine pre-treated rats receiving infusions of: corticosterone ( $n = 1$ ), mifepristone + HBC ( $n = 1$ ), and spironolactone + corticosterone ( $n = 1$ ).

An initial three-way analysis of variance (ANOVA) with one repeated measure (pre-treatment X combination of drug infusions into the ventral hippocampus X repeated measure of time) was adjusted for non-sphericity using a Greenhouse-Geisser correction and used to compare dopamine responses across time between pre-treatment- and combinations of drug infusions into the ventral hippocampus (missing values at a single time point in two saline and two amphetamine pre-treated rats precluded the inclusion of these subjects' datum from use in this ANOVA) (3.2, Fig. 3-3). Significant interactions were further analyzed by two-way ANOVAs (one repeated measure of time) or one-way ANOVA performed separately in saline- and amphetamine pre-treatment groups or across drug infusion groups. Main effects of time were followed by separate post hoc Holm-Sidak tests to identify significant changes across time within each pre-treatment group (-2 to -0.5 min time bin set as the baseline control), or between drug infusion groups.

A separate two-way ANOVA with one repeated measure (drug infusion into the ventral hippocampus X repeated measure of time) was used to determine whether changes in dopamine output differed over time as a function of Corticosterone 3-CMO : BSA infusion (compared to BSA-HBC infusion) in saline pre-treated rats (3.3, Fig. 3-4). One-way ANOVA with a repeated measure of time were also separately performed in saline pre-treated rats receiving drug infusions into the dorsoventral hippocampus and amygdala (posterior medial cortical regions) as anatomical controls, to identify whether infusions of corticosterone outside the ventral portion of the ventral hippocampus had effects on accumbal dopamine output in saline pre-treated controls (3.4, Fig. 3-5).

### 3. Results

#### *3.1. Electrode and infusion cannula placements*

Representative placements of working electrode surfaces in the nucleus accumbens shell and associated unilateral placements of drug cannula surfaces in the ventral hippocampus are drawn to scale and illustrated in Figs. 3-2, 3-4B – C, and 3-5B – C. Electrode and infusion cannula placements were similarly distributed in saline- and amphetamine pre-treated rats across all experiments and all infusion conditions. Data from rats in which the electrode placements missed the target brain region were excluded from subsequent analyses. The exception to this was when the drug infusion cannulae were found to be placed in the dorsal aspect of the ventral hippocampus or below the ventral hippocampus in the amygdala (posterior medial cortical regions, Fig. 3-5C). Data from these animals were subsequently analyzed separately as anatomical control groups (3.4).

#### *3.2. Corticosterone's effects on accumbal dopamine output differ in drug-naïve- and amphetamine withdrawn rats*

An initial three-way analysis of variance (ANOVA) with one repeated measure (pre-treatment X combination of drug infusions into the ventral hippocampus X repeated measure of time) revealed a significant interaction between pre-treatment, time, and infusion(s) ( $F(18,257) = 3.187, P < 0.0001$ ); this was followed up with Holm-Sidak post hoc two- and one way ANOVA and repeated measures ANOVAs, as reported below.

##### *3.2a. Stress-relevant concentrations of corticosterone infused into the ventral hippocampus of saline pre-treated rats enhance accumbal dopamine output*

In saline pre-treated rats, infusing vehicle + corticosterone into the ventral hippocampus significantly increased accumbal dopamine output relative to pre-infusion levels and compared to vehicle + HBC infusions, peaking at 57.5 min post-infusion (Fig. 3-3A). A two-way ANOVA (drug infusion x repeated measure of time) revealed a significant interaction between drug infusion and time ( $F(2,37) = 3.137$ ,  $P = 0.049$ ), with vehicle + corticosterone infusions resulting in a greater accumbal dopamine output at 28 – 75.5 min post-infusion as compared to vehicle + HBC control levels (Holm-Sidak  $P < 0.05$ ). A one-way repeated measures ANOVA performed in vehicle + corticosterone infused rats revealed a significant effect of time ( $F(2,21) = 4.534$ ,  $P = 0.021$ ), with vehicle + corticosterone infusions resulting in a significantly greater accumbal dopamine output at 36 – 67.5 min post-infusion relative to baseline levels (-2 to -0.5 min time bin) (Holm-Sidak,  $p < 0.05$ ). In contrast, vehicle + HBC infusions into the ventral hippocampus did not significantly alter accumbal dopamine output over time in saline pre-treated rats ( $F(4,21) = 1.025$ ,  $P = 0.410$ ).

### *3.2b. Stress-relevant concentrations of corticosterone infused into the ventral hippocampus reduce accumbal dopamine output in amphetamine withdrawal*

In rats undergoing two weeks of withdrawal from amphetamine pre-treatment, vehicle + corticosterone infusions into the ventral hippocampus significantly *reduced* accumbal dopamine output relative to pre-infusion levels and to vehicle + HBC infusions, peaking at 34.5 and 67.5 min post-infusion (Fig. 3-3A), with a significant interaction between drug infusion and time ( $F(4,68) = 3.770$ ,  $P = 0.007$ ). Here, vehicle + corticosterone infusions resulted in a reduction of accumbal dopamine output compared to vehicle + HBC infusions at 16 – 39.5 and 48 – 99.5 min post-infusion (Holm-Sidak,  $P < 0.05$ ). Further, in vehicle + corticosterone infused rats, a one-



way repeated measures ANOVA revealed a significant effect of time ( $F(3,29) = 5.454$ ,  $P = 0.004$ ), with reduced accumbal dopamine output at 56 – 85.5 min time points as compared to baseline levels (Holm-Sidak,  $P < 0.05$ ). Infusing vehicle + HBC into the ventral hippocampus was not found to alter accumbal dopamine output over time ( $F(3,19) = 0.846$ ,  $P = 0.474$ ).

*3.2c. Stress-relevant concentrations of corticosterone infused into the ventral hippocampus differentially alter accumbal dopamine output in saline- and amphetamine pre-treated rats*

To assess whether corticosterone in the ventral hippocampus alters accumbal dopamine output differently in amphetamine withdrawal (relative to saline pre-treated controls), a two-way ANOVA (pre-treatment x repeated measure of time) was performed in saline- and amphetamine pre-treated rats receiving infusions of vehicle + corticosterone into the ventral hippocampus (Fig. 3-3A). This revealed a significant interaction between pre-treatment and time ( $F(3,49) = 6.877$ ,  $P = 0.001$ ), with accumbal dopamine output differing significantly between the two pre-treatment groups at 18 – 87.5- and 94 – 101.5 min post-infusion (Holm-Sidak,  $P < 0.05$ ). When comparing the effects of vehicle + HBC infusions in saline- vs amphetamine pre-treated rats over time, there were no main effects of time ( $F(4,54) = 0.954$ ,  $P = 0.443$ ) or pre-treatment ( $F(1,13) = 0.114$ ,  $P = 0.741$ ) and no significant interactions between pre-treatment and time ( $F(4,54) = 0.904$ ,  $P = 0.471$ ).

*3.2d. Blocking either glucocorticoid- or mineralocorticoid receptors in the ventral hippocampus prevents ventral hippocampal corticosterone from altering accumbal dopamine output*

First, we assessed whether corticosterone infusions into the ventral hippocampus influence accumbal dopamine output when either glucocorticoid- or mineralocorticoid receptors are

selectively blocked with mifepristone or spironolactone (respectively) in saline pre-treated rats (Fig. 3-3B). A two-way ANOVA with one repeated measure of time, comparing the effects of mifepristone + corticosterone infusions to those observed following mifepristone + HBC infusions in saline pre-treated rats, revealed no main effects of drug infusion ( $F(1,10) = 3.853$ ,  $P = 0.078$ ) or time ( $F(3,34) = 1.257$ ,  $P = 0.306$ ) and no significant interactions between drug infusion and time ( $F(3,34) = 1.379$ ,  $P = 0.265$ ; Fig. 3-3B). Similarly, when comparing the accumbal dopamine responses to ventral hippocampus infusions of *spironolactone* + corticosterone vs spironolactone + HBC infusions (over time), there were no main effects of drug infusion ( $F(1,11) = 1.400$ ,  $P = 0.262$ ) or time ( $F(5,51) = 0.864$ ,  $P = 0.504$ ) and no significant interaction between drug infusion and time ( $F(5,51) = 1.298$ ,  $P = 0.281$ ; Fig. 3-3B).

Next, we assessed whether blocking glucocorticoid or mineralocorticoid receptors would prevent subsequent corticosterone infusions from significantly altering accumbal dopamine output *in amphetamine withdrawal* (Fig. 3-3C). In rats undergoing two weeks of withdrawal from amphetamine pre-treatment, accumbal dopamine responses to mifepristone + corticosterone infusions did not significantly differ from those observed following mifepristone + HBC infusions. A two-way repeated measures ANOVA revealed no main effects of drug infusion ( $F(1,10) = 0.850$ ,  $P = 0.378$ ) or time ( $F(4,35) = 0.881$ ,  $P = 0.474$ ), and there was no significant interaction between drug infusion and time ( $F(4,35) = 0.863$ ,  $P = 0.484$ ; Fig. 3-3C). Similarly, when comparing the accumbal dopamine responses to ventral hippocampus infusions of spironolactone + corticosterone vs spironolactone + HBC infusions (over time) there were no main effects of drug infusion ( $F(1,10) = 3.229$ ,  $P = 0.103$ ) or time ( $F(4,42) = 1.748$ ,  $P = 0.154$ ) and no significant interaction between drug infusion and time ( $F(4,42) = 0.410$ ,  $P = 0.811$ ; Fig.

3-3C). Taken together, these findings suggest that corticosterone infusions into the ventral hippocampus do not significantly alter accumbal dopamine output (over time) when glucocorticoid- or mineralocorticoid receptors are selectively blocked (in control conditions or in amphetamine withdrawal).

### *3.3. Stress-relevant concentrations of membrane-impermeable corticosterone infused into the ventral hippocampus of saline pre-treated rats fail to alter accumbal dopamine output relative to control infusions*

Within saline pre-treated rats receiving infusions of membrane impermeable corticosterone 3-CMO : BSA or its vehicle (BSA-HBC) into the ventral hippocampus, drug infusion was not found to have a significant effect on accumbal dopamine output ( $F(1,12) = 0.00555$ ,  $P = 0.942$ ), and there was no main effect of time ( $F(68,816) = 1.098$ ,  $P = 0.280$ ) nor a significant interaction between time and infusion ( $F(68,816) = 0.951$ ,  $P = 0.590$ ) (Fig. 3-4). Note: this data set failed the Shapiro-Wilk normality test and the Brown-Forsyth equal variance test, though similar findings were observed when a Greenhouse-Geisser correction was applied to correct for non-sphericity (Greenhouse-Geisser correction was not used in the above statistics, but was run to assess the accuracy of the above statistics given their failure to meet assumptions of normality and equal variance).

### *3.4. Location of corticosterone infusion differentially impacts accumbal dopamine output in saline pre-treated controls*

To determine the specificity of corticosterone infusions in the ventral portion of the ventral hippocampus, we also tested whether vehicle + corticosterone infusions made into the

*dorsoventral* hippocampus ( $n = 5$ ) or below the ventral hippocampus into the posterior medial cortical regions of the amygdala ( $n = 4$ ) (including the amygdalohippocampal area (AHiPM,  $n = 1$ ), posteriomedial cortical area (PMCO,  $n = 2$ ), and amygdalaopiriform atransitional area (APir,  $n = 1$ )) produced alterations in accumbal dopamine output (Fig. 3-5). A one-way repeated measures ANOVA revealed that vehicle + corticosterone infusions into the dorsoventral hippocampus did produce a significant increase in accumbal dopamine output over time ( $F(65, 260) = 1.974, P < 0.001$ ). However, no specific time period was greater over time when multiple comparisons were assessed pairwise or vs. pre-infusion controls (Holm Sidak,  $P > 0.05$ ). When assessing the effects of vehicle + corticosterone infusions made into the posterior medial cortical regions of the amygdala on accumbal dopamine output over time, a one-way ANOVA failed the Shapiro-Wilk normality test (but passed the Brown-Forsythe Equal Variance test). Similar to the effects of dorsoventral hippocampal infusions, infusions into the posterior medial cortical amygdala did produce a significant increase in accumbal dopamine output over time ( $F(60, 180) = 1.860, P < 0.001$ ), but no specific time period was greater over time when multiple comparisons were assessed pairwise or vs. pre-infusion controls (Holm Sidak,  $P > 0.05$ ).

## **4. Discussion**

### *4.1. Ventral hippocampal corticosterone enhances accumbal dopamine output in controls*

Corticosterone is thought to be excitatory in the ventral hippocampus and can induce rapid glutamate release *in vitro* (Bekkers & Stevens, 1989; Karst *et al.*, 2005). Excitation in the ventral hippocampus enhances accumbal dopamine output (Blaha *et al.*, 1997; Karst *et al.*, 2005; Barr *et al.*, 2014; Taepavarapruk *et al.*, 2014) (Fig. 1-3). Therefore, we hypothesized that infusing corticosterone into the ventral hippocampus of saline pre-treated rats would rapidly enhance

accumbal dopamine release. As predicted, accumbal dopamine levels rapidly increased after vehicle + corticosterone infusions (3.2a, Fig. 3-3A). This suggests that the ventral hippocampal corticosterone stress response (Droste *et al.*, 2008; Bray *et al.*, 2016) may contribute to the ability of stress exposure to enhance reward salience and motivate goal-oriented behavior in control conditions (Floresco, 2014; Hollon *et al.*, 2015; Berridge & Robinson, 2016). Furthermore, acute psychostimulant exposure is known to enhance corticosterone secretion (Knych & Eisenberg, 1979; Swerdlow *et al.*, 1993; Bayer *et al.*, 1995; Zuloaga *et al.*, 2014). Therefore, our findings also suggest that corticosterone in the ventral hippocampus could provide a mechanism that contributes to positive reinforcement of initial psychostimulant use (Robinson & Berridge, 2000). A variety of systemic mechanisms have been proposed that may enable excitatory stimulation in the ventral hippocampus to enhance accumbal dopamine output in control conditions (Fig. 1-3) (Blaha *et al.*, 1997; Legault *et al.*, 2000; Floresco *et al.*, 2001; Taepavarapruk *et al.*, 2008; Belujon & Grace, 2011; Tye, 2012; Barr *et al.*, 2014; Taepavarapruk *et al.*, 2014). Possible receptor mechanisms that enable this response are addressed below (4.6).

#### *4.2. Ventral hippocampal corticosterone reduces accumbal dopamine output in protracted amphetamine withdrawal*

In amphetamine pre-treated rats, corticosterone infusions into the ventral hippocampus produced a biphasic *reduction* in accumbal dopamine output (3.2b, Fig. 3-3A). These findings suggest that accumbal dopamine output responds differently to ventral hippocampal corticosterone in amphetamine withdrawal (3.2c). The mechanistic findings thought to mediate this differential response are addressed below (4.7). Overall, these findings suggest that by reducing accumbal dopamine output in protracted amphetamine withdrawal, the ventral hippocampal corticosterone

stress response may contribute to dysphoric states that are associated with accumbal dopamine deficiency and that predict drug-taking behavior and relapse (Weise-Kelly & Siegel, 2001; Ungless *et al.*, 2004; Sinha, 2007; Paliwal *et al.*, 2008; Roitman *et al.*, 2008; Wheeler *et al.*, 2008; Brischoux *et al.*, 2009; Wheeler & Carelli, 2009; Wheeler *et al.*, 2011; Robinson *et al.*, 2014; Twining *et al.*, 2014; Hurley *et al.*, 2017; Haake *et al.*, 2018). Therefore, our findings support an opponent-process theory of addiction (Fig. 1-1), in which blunted dopamine reward responses and enhanced glucocorticoid stress responses contribute to negative reinforcement of drug-taking (Koob & Le Moal, 2008a; b). Previous research has focused on the role of corticotrophin releasing factor (CRF) in the amygdala as contributing to the disruption of reward processes thought to prompt addiction (Koob & Le Moal, 2008a; b; Koob, 2009; Koob & Zorrilla, 2010; Zorrilla *et al.*, 2014). However, our findings support a *bona fide* role for corticosterone in the ventral hippocampus as contributing to the dysregulated opponent processes thought to negatively reinforce drug dependence (Koob & Le Moal, 2008a; b).

#### *4.3. The Rapid Onset of Ventral Hippocampal Corticosterone's Effects on Accumbal Dopamine Output Are Consistent with Non-Genomic Mechanisms*

Cytosolic receptor translocation to the nucleus and subsequent transcription are thought to take between ~10 – 30 min and ~5 – 120 min respectively (Haller *et al.*, 2008). Therefore, effects with a lag time of less than 15 min have been suggested to be mediated by non-genomic mechanisms (Stahn *et al.*, 2007; Stahn & Buttgerit, 2008; Groeneweg *et al.*, 2012). Membrane-bound receptors that modulate membrane ion channels have also been suggested to have a receptor response latency of equal to or less than 30 min (Prager & Johnson, 2009). Although our observed effects did not reach significance until 28 - 36 min in control conditions and 16 - 48

min in amphetamine withdrawal, these effects were found to *begin* almost immediately after infusions (3.2a – b, Fig. 3-3A), suggesting that non-genomic mechanisms may be involved at least initially.

Notably, the biphasic reduction in accumbal dopamine release observed in amphetamine withdrawal (3.2b, Fig. 3-3A) may represent two temporally different responses mediated by two separate mechanisms. The first reduction in accumbal dopamine output onset rapidly and reached significance at 16 min post-infusion, suggesting it may be non-genomic. The second reduction in accumbal dopamine release reached significance relative to vehicle + HBC- *and pre-infusion* levels at 48 – 56 min post-infusion. The presence of the initial reduction makes it difficult to surmise when the effects of the second peak reduction begin. Therefore, it is possible that this second peak is mediated by a genomic pathway, though non-genomic pathways cannot be excluded.

#### *4.4. Stress-relevant concentrations of membrane-impermeable corticosterone in the ventral hippocampus fail to alter accumbal dopamine output*

Corticosterone's non-genomic effects are traditionally attributed to membrane-associated mechanisms (Fig. 1-5) (Tasker *et al.*, 2006; Groeneweg *et al.*, 2012; Barr *et al.*, 2017; Joels & de Kloet, 2017). However, cytosolic mineralocorticoid and glucocorticoid receptors and receptor complexes can also induce non-genomic effects (Tumlin *et al.*, 1997; Croxtall *et al.*, 2002; Horvath & Wanner, 2006; Liu *et al.*, 2010). Surprisingly, we found that isolating corticosterone's actions to the cell membrane by infusing membrane-impermeable corticosterone 3-CMO : BSA into the ventral hippocampus prevented ventral hippocampal corticosterone from altering

accumbal dopamine output in saline pre-treated controls (3.3, Fig. 3-4A). This suggests that corticosterone's effects on accumbal dopamine output cannot be attributed entirely to membrane-associated mechanisms, and must rely at least partly on cytosolic means. This conclusion does not preclude the possibility that ventral hippocampal corticosterone alters accumbal dopamine output through non-genomic processes. For example, corticosterone activation of non-genomic cytosolic glucocorticoid receptors has been directly demonstrated to rapidly and reversibly inhibit ATP-induced currents in rat dorsal root ganglion cultures (Liu *et al.*, 2008; Liu *et al.*, 2010). This suggests that non-genomic cytosolic glucocorticoid receptors may be involved in mediating the rapid of ability of ventral hippocampal corticosterone to alter accumbal dopamine release (as observed in Fig. 3-3A). It is also possible that concomitant activation of *both* membrane-bound *and* cytosolic mechanisms may be required for ventral hippocampal corticosterone to alter accumbal dopamine release. This possibility would suggest that membrane mechanisms may *contribute* to the observed response (Fig. 3-3) but may not be sufficient to induce it on their own. Therefore, blocking corticosterone's access to the cytosol would block its effects, as we observed. Overall, our findings suggest that in the ventral hippocampus, corticosterone can induce rapid effects through mechanisms that are at least partly cytosolic.

#### *4.5. Selectively blocking either mineralocorticoid or glucocorticoid receptors in the ventral hippocampus prevents hippocampal corticosterone from altering accumbal dopamine release*

We hypothesized that isolating corticosterone's effects to one particular receptor would reveal the functional effects of the other receptor on accumbal dopamine output *in vivo*. Surprisingly, selectively blocking either glucocorticoid- or mineralocorticoid receptors in the ventral hippocampus prevented ventral hippocampal corticosterone from altering accumbal dopamine



output in both control conditions and in amphetamine withdrawal (3.2d, Figs. 3-3B and 3-3C). This suggest that neither glucocorticoid- nor mineralocorticoid receptors in the ventral hippocampus can sufficiently enable ventral hippocampal corticosterone to alter accumbal dopamine output independently of one another. This suggestion derives from the assumption that blocking one receptor would reveal the effects of the other receptor, which appear to be nullified. These findings also suggest that activation of *both* receptor subtypes may be *required* to produce this effect, since independently blocking *either* receptor subtype blocked the observed effects. Therefore, we propose that corticosterone in the ventral hippocampus alters accumbal dopamine output through mechanisms that require concomitant cooperative or additive activation of *both* glucocorticoid *and* mineralocorticoid receptors in the ventral hippocampus (3.2d, 4.5) that are at least partly cytosolic (3.3, 4.4) and are likely to be at least partly non-genomic (4.3), as discussed in 4.6.

*4.6. Ventral hippocampal corticosterone may alter accumbal dopamine output through cooperative and compulsory activation of excitatory membrane mineralocorticoid receptors and disinhibitory cytosolic glucocorticoid receptors*

Overall, our findings suggest that isolating corticosterone activation to one specific receptor subtype cannot adequately reveal all the specific effects of the other receptor subtype on accumbal dopamine release. Therefore, the following conclusions rely on existing information about glucocorticoid and mineralocorticoid receptors, which are derived primarily from studies conducted *in vitro* and *ex vivo* in a variety of limbic tissues that all differ morphologically, cyto- and chemoarchitecturally, and functionally (Di *et al.*, 2003; Bannerman *et al.*, 2004; Karst *et al.*,

2005; Maggio & Segal, 2007; Hu *et al.*, 2010; Liu *et al.*, 2010; Strange *et al.*, 2014; Di *et al.*, 2016).

The ventral hippocampus is known to have an extensive interneuronal network responsible for regulating local excitation (Fig. 1-4D, 4-1)(Freund & Buzsaki, 1996; Leranth & Hajszan, 2007; Chamberland & Topolnik, 2012). This network includes interneurons that specifically target other interneurons (*interneuron specific interneurons*, IS-IS)(Freund & Buzsaki, 1996; Isaacson & Scanziani, 2011; Chamberland & Topolnik, 2012). Electrophysiology studies suggest that pre- and postsynaptic membrane mineralocorticoid receptors can rapidly and reversibly potentiate glutamate release in the hippocampus (Fig. 1-4B) (Bekkers & Stevens, 1989; Karst *et al.*, 2005). Non-genomic glucocorticoid receptors are thought to regulate interneuronal inhibition (Zeise *et al.*, 1992), which may occur in part through their recruitment of membrane permeable retrograde signaling mechanisms such as nitric oxide (NO, a gas) and 2-arachidonoylglycerol (2-AG, an ester/lipid) (Figs. 1-4C-D and 4-1) (Di *et al.*, 2003; Hu *et al.*, 2010; Di *et al.*, 2016). Furthermore, in contrast to non-genomic mineralocorticoid receptors, which are thought to be localized to the postsynaptic membrane (Karst *et al.*, 2005), non-genomic glucocorticoid receptors can be cytosolic (Hu *et al.*, 2010; Liu *et al.*, 2010) and can induce diffuse effects that are not restricted by cell membranes.

Based on the above information, we propose that the ventral hippocampus' glutamatergic efferents responsible for stimulating accumbal dopamine release may be under tonic inhibition in basal conditions (Fig. 4-1). We propose cytosolic non-genomic glucocorticoid receptor activation is required to disinhibit these neurons and activation of non-genomic membrane

mineralocorticoid receptors is also required to potentiate glutamate release *when these neurons are disinhibited* (Figs. 4-1, 1-4). Therefore, independently activating mineralocorticoid receptors *could* rapidly potentiate glutamate release from the ventral hippocampus's glutamate projection neurons that stimulate accumbal dopamine output (Karst *et al.*, 2005; Prager *et al.*, 2010; Chaouloff & Groc, 2011; Pasricha *et al.*, 2011). However, we propose this potentiation may be suppressed by presynaptic GABAergic interneuron inhibition without concomitant glucocorticoid-receptor-mediated disinhibition. Likewise, independently activating glucocorticoid receptors may have a disinhibitory effect locally (Zeise *et al.*, 1992; Di *et al.*, 2003; Hu *et al.*, 2010; Liu *et al.*, 2010; Di *et al.*, 2016). However, this effect may not be sufficient to significantly alter accumbal dopamine release in the absence of mineralocorticoid-receptor-induced excitation.

Similarly, isolating corticosterone's effects to the cell membrane may activate excitatory membrane mineralocorticoid receptors, but these excitatory effects may be silenced by top-down GABAergic interneuron inhibition without concomitant activation of cytosolic glucocorticoid-receptor-mediated disinhibition. Moreover, membrane glucocorticoid receptors have been proposed to be insensitive to mifepristone (Venero & Borrell, 1999; Li *et al.*, 2014). We found that the effect of ventral hippocampal corticosterone on accumbal dopamine output *was* blocked by mifepristone (Fig. 3-3B-C). Therefore, this finding further implicates glucocorticoid receptors *in the cytosol* (rather than in the membrane) as contributing to the observed effects on accumbal dopamine output (Fig. 3-3). Overall, we conclude that ventral hippocampal corticosterone alters accumbal dopamine release through compulsory cooperative activation of excitatory membrane mineralocorticoid receptors and disinhibitory cytosolic glucocorticoid receptors. This conclusion

nicely accommodates our present findings, which suggest that in control conditions, corticosterone in the ventral hippocampus enhances accumbal dopamine output (3.2a) through mechanisms that appear to be at least partly non-genomic (4.3) and yet require cytosolic access (3.3, 4.4) *and* activation of both mineralocorticoid- and glucocorticoid receptors (3.2d, 4.5).

However, this working theory falls short in explaining how corticosterone in the ventral hippocampus can *reduce* accumbal dopamine output in amphetamine withdrawal (3.2b).

We found that selectively blocking either glucocorticoid or mineralocorticoid receptors in the ventral hippocampus (prior to corticosterone infusions) produced the same functionally nullified effects on accumbal dopamine output in control conditions and in withdrawal (3.2d). This makes it difficult to discern whether either receptor subtype has a functionally different response to corticosterone activation during withdrawal. We propose that the same compulsory cooperation between cytosolic glucocorticoid receptor disinhibition and membrane mineralocorticoid receptor excitation described above (4.6) may persist at the receptor level within the ventral hippocampus in both control conditions and in amphetamine withdrawal (however, see discussion in chapter 4 on the potential role of deficient serotonin signaling). Therefore, the *reducing* effects of ventral hippocampal corticosterone on accumbal dopamine output in protracted amphetamine withdrawal may be driven instead by enhanced *inhibitory* tone within the hippocampal-mesolimbic circuit during protracted withdrawal, as addressed in Fig. 4-1B and below (4.7).

*4.7. Enhanced inhibitory tone within the mesolimbic system may contribute to the differential effects of ventral hippocampal corticosterone on accumbal dopamine output in withdrawal*

Like the hippocampus, the nucleus accumbens and ventral tegmental area have extensive inhibitory networks that regulate activity of their principal cells with fine-tuned precision (Tepper & Bolam, 2004; Tepper *et al.*, 2008; Creed *et al.*, 2014; Burke *et al.*, 2017; Scudder *et al.*, 2018). Recently, a population of disynaptic glutamatergic efferents from the ventral hippocampus was identified within the medial nucleus accumbens shell (Scudder *et al.*, 2018). The disynaptic terminals synapse onto excitatory D<sub>1</sub>- and inhibitory D<sub>2</sub> medium spiny neurons (MSNs) and onto feed-forward interneurons and thus regulate the excitation/inhibition tone of MSNs within the nucleus accumbens shell (Scudder *et al.*, 2018). These disynaptic efferents are thought to ultimately maintain a “backdrop” of inhibition within the accumbal shell (Scudder *et al.*, 2018). In this same study, 5 days of repeated cocaine exposure was found to shift the excitation/inhibition balance toward excitation through terminal changes in synaptic preferences. Importantly, dopamine and MSN excitation and inhibition within the medial nucleus accumbens shell can induce direct- and indirect feedback onto medial ventral tegmental area dopamine cell activity to influence accumbal shell dopamine output in turn (Figs. 1-3, 4-1)(Floresco *et al.*, 2001; Pignatelli & Bonci, 2018; Yang *et al.*, 2018). This suggests that the increased excitatory tone in the nucleus accumbens shell observed in acute psychostimulant withdrawal (Scudder *et al.*, 2018) could result in excitatory output from the ventral hippocampus having a *super-excitatory* effect on accumbal dopamine release during acute withdrawal, as observed by Barr *et al.* (2014) (see also discussion in Appendix 2).

*Protracted* amphetamine withdrawal is known to alter synaptic connections in the nucleus accumbens shell (Scofield *et al.*, 2016). Therefore, it is possible that repeated amphetamine exposure shifts the excitatory/inhibitory balance in the nucleus accumbens shell from one of

*excitation* in acute withdrawal (Scudder *et al.*, 2018) to one favoring *inhibition* in protracted withdrawal. This could result in excitatory output from the ventral hippocampus having an *excitatory* effect on MSN activity (Scudder *et al.*, 2018) *and on subsequent VTA dopamine output into the nucleus accumbens shell* (Pignatelli & Bonci, 2018; Yang *et al.*, 2018) in control conditions and a *super-excitatory* effect on accumbal dopamine release in *acute* withdrawal, but an *inhibitory* or *reducing* effect on accumbal dopamine output during *protracted* psychostimulant withdrawal. Therefore, a shift to an enhanced *inhibitory* tone in the nucleus accumbens shell during *protracted* psychostimulant withdrawal (Fig. 4-1B) could explain how corticosterone in the ventral hippocampus can have an excitatory effect on local and accumbal glutamate release in both control conditions and in amphetamine withdrawal, yet *reduce* accumbal shell dopamine output during *protracted psychostimulant withdrawal* (3.2b).

#### *4.8. Location of corticosterone infusion differentially impacts accumbal dopamine output*

The dorsal aspect of the ventral hippocampus has high expression of glucocorticoid- and mineralocorticoid receptors (Reul & de Kloet, 1985; 1986), but projects to the nucleus accumbens core rather than to the shell (Strange *et al.*, 2014). Although information on the functional connectivity of the posterior medial cortical regions of the amygdala is limited, existing literature suggests functional connectivity to the ventral hippocampus and other regions of the amygdala, but not to the nucleus accumbens shell (Sah *et al.*, 2003; Groeneweg *et al.*, 2012; Schmitt *et al.*, 2012; Zorrilla & Koob, 2013; Salgado & Kaplitt, 2015; Belujon *et al.*, 2016). Therefore, we were surprised that corticosterone infusions into the dorsoventral hippocampus or posterior medial cortical regions of the amygdala *did* significantly alter accumbal dopamine output over time (3.4, Fig. 3-5). However, these findings were non-specific

in both regions, and no particular time period was found to differ significantly over time when compared with pre-infusion levels, or in pairwise comparisons (Fig. 3-5A). Given the lack of any known projections from the dorsoventral hippocampus or posterior medial cortical regions of the amygdala to the nucleus accumbens shell (Schmitt *et al.*, 2012; Gutierrez-Castellanos *et al.*, 2014; Strange *et al.*, 2014), the non-specific effects of corticosterone infusions on accumbal dopamine output observed in these regions most likely resulted from corticosterone diffusion into the ventral sub-regions of the hippocampus. However, future research could benefit from further testing whether corticosterone in the amygdala can also alter accumbal dopamine output, particularly in the basolateral- or central regions that are known to respond to corticosterone and project to the nucleus accumbens shell (Floresco *et al.*, 1998; Herman *et al.*, 2005; Karst *et al.*, 2010).

## **5. Conclusions**

Here we demonstrate for the first time that stress-relevant concentrations of corticosterone in the ventral hippocampus can significantly enhance accumbal dopamine output in control conditions, and reduce accumbal dopamine output in protracted amphetamine withdrawal, with the accumbal dopamine response differing significantly in withdrawal. Our findings suggest that the ability of corticosterone in the ventral hippocampus to alter accumbal dopamine output requires cytosolic access as well as activation of both mineralocorticoid and glucocorticoid receptors that are likely to be at least partly mediated by non-genomic mechanisms. Thus, we propose that the ability of corticosterone in the ventral hippocampus to alter accumbal dopamine output requires cooperative activation of excitatory membrane mineralocorticoid receptors and cytosolic glucocorticoid-receptor-mediated disinhibition to produce excitatory output onto the mesolimbic

dopamine circuit. Enhanced inhibitory tone within the meso-hippocampal system may explain the differential effects of ventral hippocampal corticosterone on accumbal dopamine release (3.2c, Fig. 3-3A), resulting in excitatory output from the ventral hippocampus having an *excitatory* effect on accumbal dopamine output in control conditions, but an *inhibitory* or *reducing* effect during amphetamine withdrawal.

Overall, our findings suggest that the ventral hippocampal corticosterone stress response may provide a mechanism that enables stress to enhance incentive salience and promote goal-oriented behavior in control conditions. This response may also contribute to positive reinforcement of initial drug exposure and to the dysphoric states thought to negatively reinforce drug dependence and relapse during withdrawal. Our findings thus support an opponent process theory of addiction (Fig. 1-1), in which blunted dopamine reward responses and enhanced corticosterone stress responses are thought to mediate the transition from positively reinforced drug taking to negatively reinforced drug dependence. Furthermore, our findings emphasize a *bona fide* role for the hippocampal corticosterone system as contributing to dysregulation of reward and anti-reward circuitry thought to potentiate relapse during drug withdrawal (Koob & Le Moal, 2008a; b).

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### Figure Legends:

**Figure 3-1. Sample *in vivo* voltammetry recording used to confirm dopamine detection and ensure selective oxidation of dopamine (excluding electrical potentials that elicit norepinephrine and ascorbic acid oxidation) in chronoamperometry.** The dashed vertical lines indicate a sample 300 mV range of electrical potentials that was selected for each subject used in this study (based on voltammograms obtained separately for each individual subject) that encompasses dopamine oxidation only, and excludes electrical potentials that elicit norepinephrine and ascorbic acid oxidation. This 300 mV “ramp interval” is subsequently applied repetitively to the working electrode during chronoamperometry in brief (1 s) pulses at 30 s intervals to ensure that only dopamine oxidation current is induced and measured, without interference from other oxidizing species.

**Figure 3-2. Representative diagrams of carbon paste electrode placements in the medial nucleus accumbens shell (bregma +1.60 mm) (left) and infusion cannula placements in the ventral subiculum and ventral dentate gyrus regions of the hippocampus (bregma -5.80 mm) (right) in saline- and amphetamine pre-treated rats,** corresponding with results shown in Figs. 3-3A, B, and C. **A)** Histology corresponding with results shown in Fig. 3-3A, in which saline- and amphetamine pre-treated rats received infusions of vehicle + corticosterone (shown in red for saline pre-treatment,  $n = 11$ ; shown in blue for amphetamine pre-treatment,  $n = 10$ ) or vehicle + HBC (saline pre-treatment shown in pink,  $n = 9$ ; amphetamine pre-treatment shown in light blue,  $n = 9$ ). **B and C)** Histology corresponding with Figs. 3-3B and 3-3C respectively, conducted in saline- (**B**) and amphetamine (**C**) pre-treated rats receiving infusions of mifepristone- or spironolactone + corticosterone or HBC (mifepristone + corticosterone shown in

dark green,  $n = 6/\text{group}$ ; mifepristone + HBC shown in light green,  $n = 6/\text{group}$ ; spironolactone + corticosterone shown in dark purple,  $n = 6-7/\text{group}$ ; spironolactone + HBC shown in light purple;  $n = 7/\text{group}$ ) with vehicle + corticosterone or HBC control placements (as displayed in A) shown for reference. Figures adapted from Paxinos and Watson (1998).

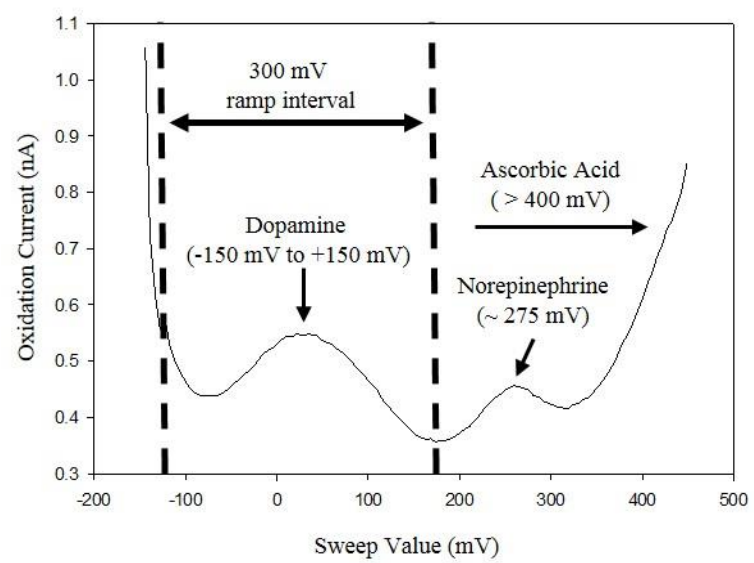
**Figure 3-3. Corticosterone infusions into the ventral hippocampus differentially alter accumbal dopamine output in saline- and amphetamine pre-treated rats (A) and blocking either glucocorticoid- or mineralocorticoid receptors in the ventral hippocampus blocks the effects in both conditions (B-C).** These line graphs compare average nucleus accumbens dopamine oxidation current (mean  $\pm$  SEM nA) over time in: **A)** saline- and amphetamine pre-treated rats receiving 0.5  $\mu\text{L}$  ventral hippocampus infusions of VEH + CORT (SAL:  $n = 11$ ; AMP:  $n = 10$ ) or VEH + HBC (SAL:  $n = 9$ ; AMP:  $n = 9$ ); and **B)** saline- or **C)** amphetamine pre-treated rats receiving ventral hippocampus infusions of MIF + CORT (SAL:  $n = 6$ ; AMP:  $n = 6$ ), MIF + HBC (SAL:  $n = 6$ ; AMP:  $n = 6$ ), SPIR + CORT (SAL:  $n = 6$ ; AMP:  $n = 7$ ), or SPIR + HBC (SAL:  $n = 7$ ; AMP:  $n = 7$ ) (with tracings for VEH + CORT infusions from Figure 3-3A shown as dotted lines). \* $P < 0.05$  vs -2 to -0.5 min control time (within pre-treatment). # $P < 0.05$  vs VEH + HBC (within pre-treatment). § $P < 0.05$  vs pre-treatment. SAL: Saline Pre-treatment; AMP: Amphetamine Pre-treatment; VEH: Vehicle; SPIR: Spironolactone; MIF: Mifepristone; CORT: Corticosterone.

**Figure 3-4. Stress-relevant concentrations of membrane-impermeable corticosterone infused into the ventral hippocampus of saline pretreated rats fail to alter accumbal dopamine output relative to vehicle infusions.** **A)** Line graph comparing average nucleus accumbens dopamine oxidation current (Mean  $\pm$  SEM nA) over time in saline pre-treated rats receiving ventral hippocampus infusions of corticosterone 3-CMO : BSA (BSA-CORT, black,  $n = 8$ ) or BSA-HBC (gray,  $n = 7$ ), with tracing for vehicle + corticosterone (VEH + CORT) infusions from Figure 3-3A shown as dotted lines for reference. **B - C)** Representative diagrams of carbon paste electrode placements in the medial nucleus accumbens shell (bregma 1.60 mm) (**B**) and infusion cannula placements in the ventral hippocampus (bregma -5.80 mm) (**C**). Histology for vehicle + corticosterone or HBC infusions (as displayed in Fig. 3-3A) shown as partly shaded plots for reference. Figures adapted from Paxinos and Watson (1998).

**Figure 3-5. Location of corticosterone infusion differentially impacts accumbal dopamine output in saline pretreated controls.** **A)** Line graph comparing average nucleus accumbens dopamine oxidation current (mean  $\pm$  SEM nA) over time in saline pre-treated rats receiving infusions of vehicle + corticosterone into the dorsoventral hippocampus (gray,  $n = 5$ ) or posterior medial cortical regions of the amygdala (black,  $n = 4$ ) with tracing for accumbal dopamine output following vehicle + corticosterone infusions into the ventral hippocampus (vHipp) from Figure 3-3A shown as dotted line for reference. \*\*significant effect of time observed when vehicle + corticosterone infusions were made into the dorsoventral hippocampus or into the posterior medial cortical regions of the amygdala ( $P < 0.001$ ), with no significant differences observed at any specific time points, pairwise or vs. pre-infusion levels ( $P > 0.05$ , Holm Sidak). **B - C)** Representative diagrams of carbon paste electrode placements in the medial nucleus

accumbens shell (bregma +1.60 mm) and corresponding infusion cannula placements in the dorsoventral hippocampus (black) and amygdala (gray) (bregma -5.80 mm) with histology for vehicle + corticosterone or HBC infusions (as displayed in Fig. 3-3A) shown as partly shaded plots for reference. Within the amygdala, infusions were made into the amygdalohippocampal area (AHiPM,  $n = 1$ ), posteromedial cortical area (PMCO,  $n = 2$ ), and amygdalaopiriform atransitional area (APir,  $n = 1$ ). Figures adapted from Paxinos and Watson (1998).

**Figure 3-1**



**Figure 3-2**

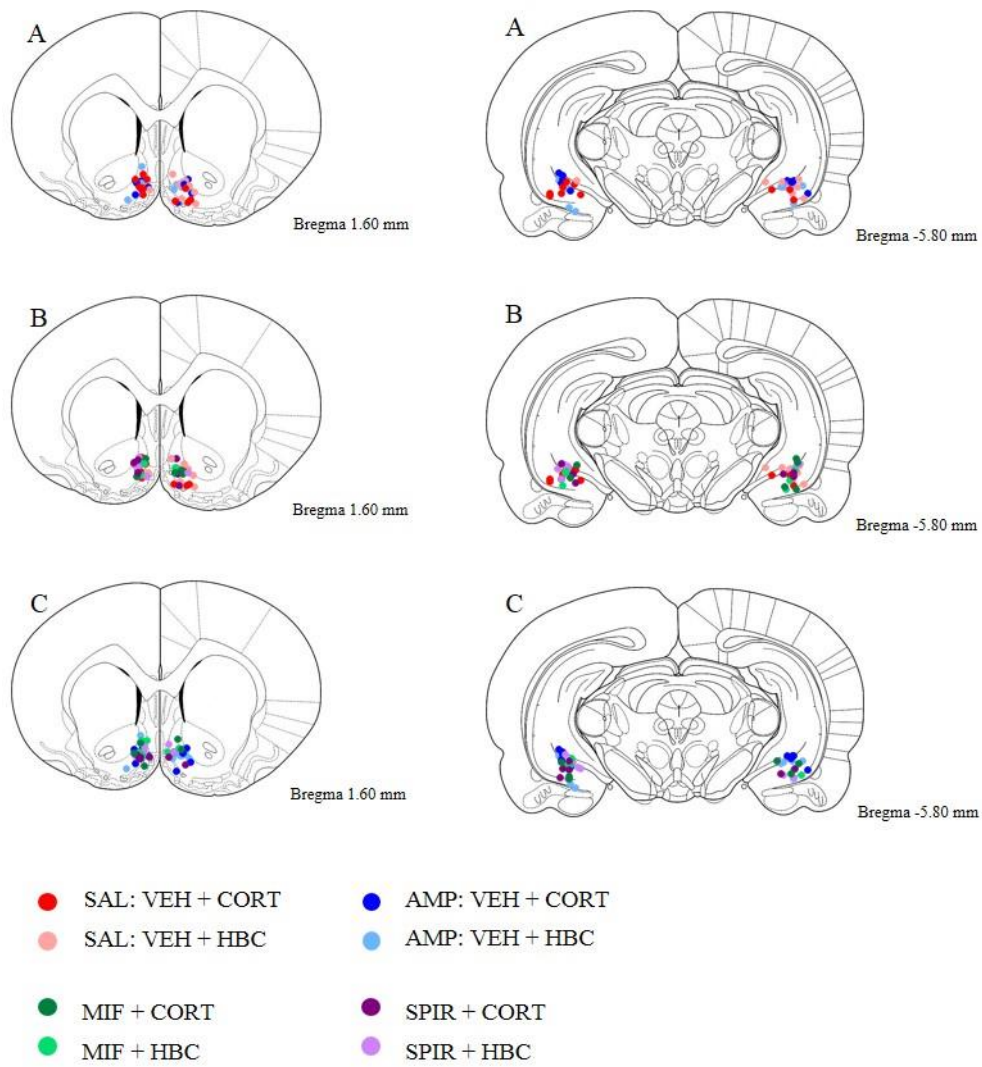
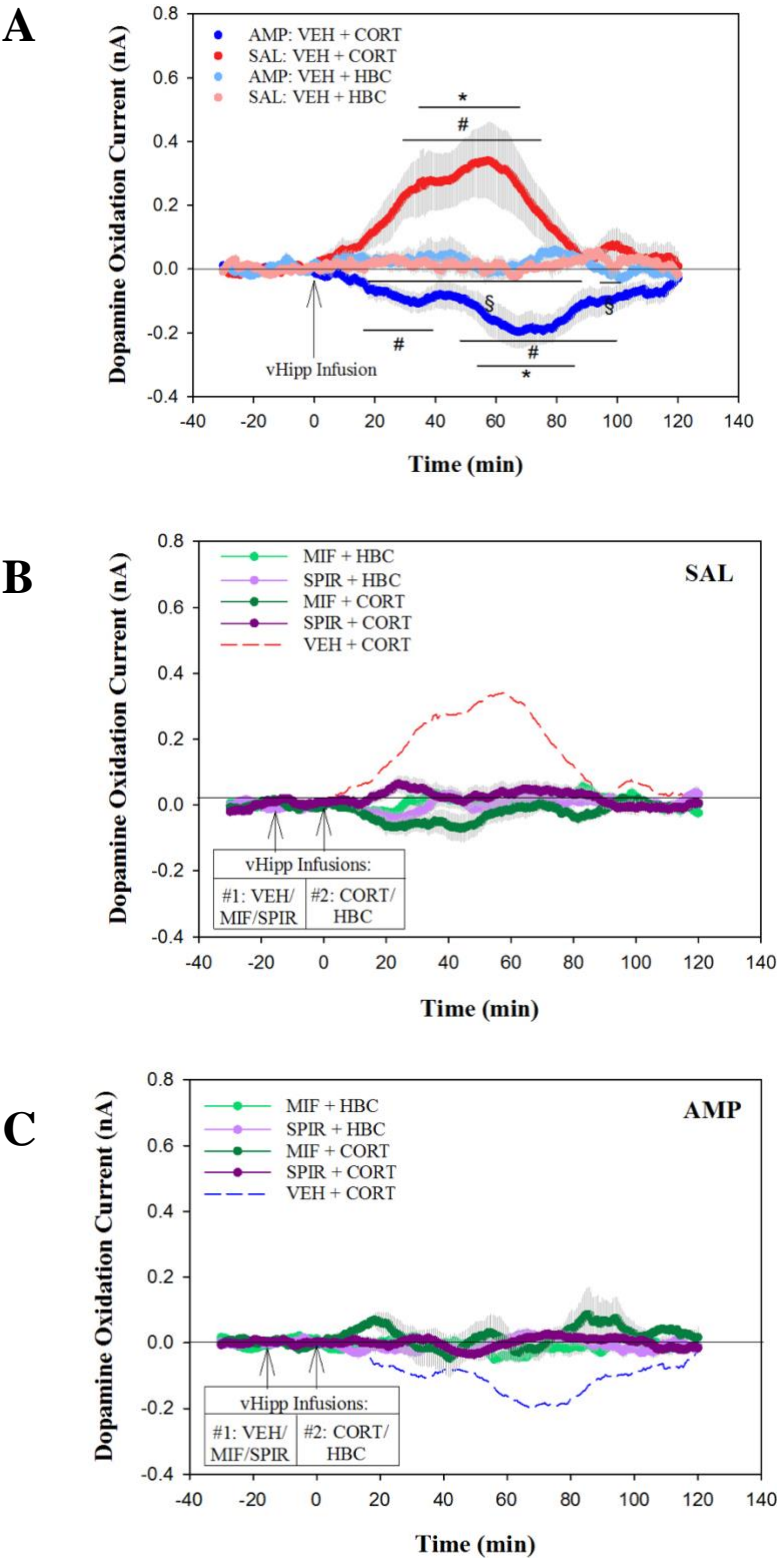


Figure 3-3



**Figure 3-4**

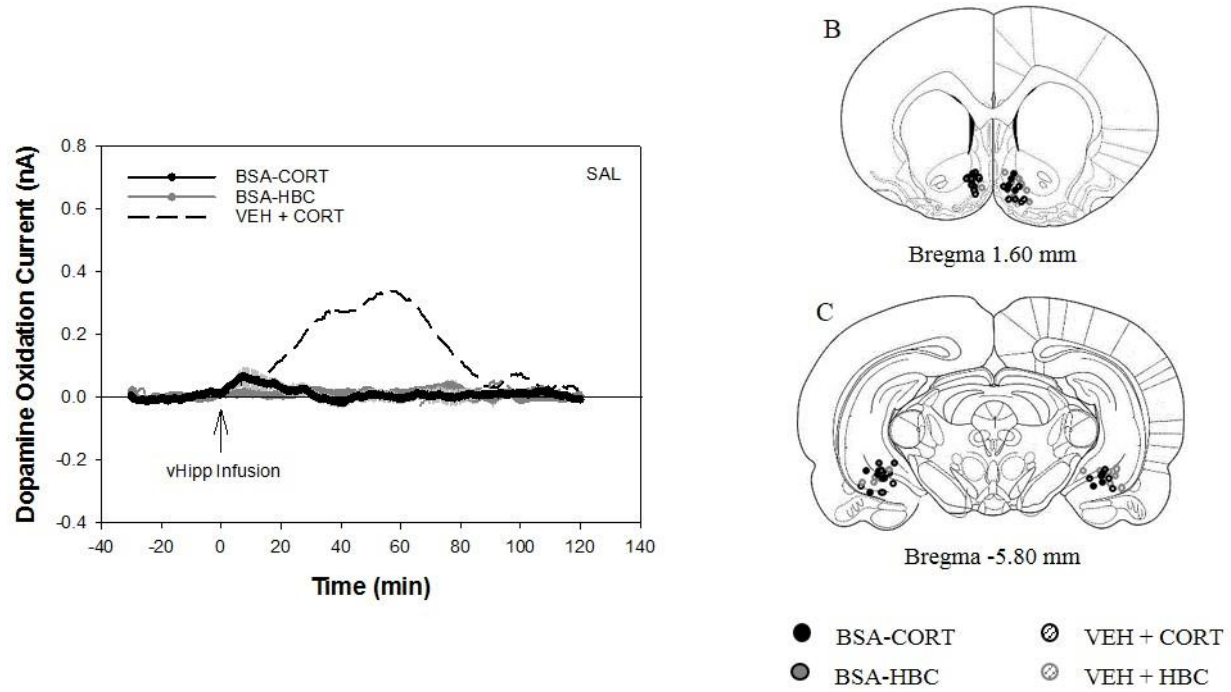
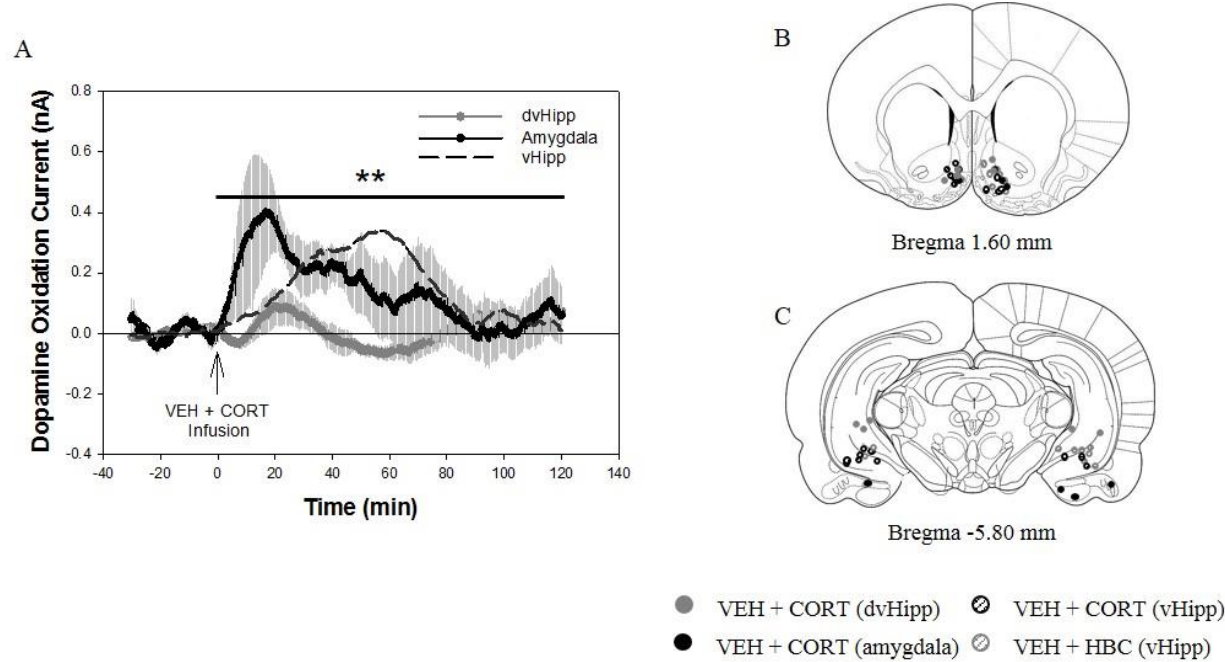




Figure 3-5



## Chapter 4: General discussion

Amphetamine withdrawal is a DSM-5 disorder with high relapse rates and no effective treatments for relapse prevention (Gossop, 2009; APA, 2013; Heal *et al.*, 2013; Uddin *et al.*, 2017; NIDA, 2018). Relapse in amphetamine withdrawal is thought to be driven by alleviation of negative affect states (Gossop, 2009) that include enhanced anxiety, dysphoria, and behavioral responses to stress (Russig *et al.*, 2006; Kitanaka *et al.*, 2008; Li *et al.*, 2014; Tu *et al.*, 2014; Barr *et al.*, 2017). The ventral hippocampus is a brain region associated with regulating emotion, stress responses, and drug behaviors through monoamine (serotonin), glucocorticoid (cortisol/corticosterone) and excitatory/inhibitory signaling mechanisms (Taepavarapruk & Phillips, 2003; Bannerman *et al.*, 2004; Herman & Mueller, 2006; Fanselow & Dong, 2010; Koob & Volkow, 2010; Belujon & Grace, 2011; Barr *et al.*, 2014; Taepavarapruk *et al.*, 2014; Tu *et al.*, 2014; Barr *et al.*, 2017).

The negative affect states observed in amphetamine withdrawal are associated with dysregulations in glucocorticoid and monoamine interactions in the ventral hippocampus (Barr & Forster, 2011; Barr *et al.*, 2014; Li *et al.*, 2014; Tu *et al.*, 2014; Bray *et al.*, 2016; Barr *et al.*, 2017). For example, corticosterone in the ventral hippocampus can induce serotonin release (Barr & Forster, 2011), which reduces anxiety (Tu *et al.*, 2014). This response is abolished in acute amphetamine withdrawal, when glucocorticoid receptor expression is reduced (Barr & Forster, 2011). The glucocorticoid-receptor-mediated serotonin stress response is also abolished in *protracted* amphetamine withdrawal, when stress-induced behavioral responses remain enhanced (Li *et al.*, 2014).

Corticosterone can also regulate *excitation and inhibition* in the hippocampus. This may involve the ability of glucocorticoid receptors to recruit retrograde and serotonin signaling (Zeise *et al.*, 1992; Morales *et al.*, 1996; Di *et al.*, 2003; Maggio & Segal, 2009; Hu *et al.*, 2010; Barr & Forster, 2011; Di *et al.*, 2016). Excitation in the ventral hippocampus can enhance accumbal dopamine release and reinstate extinct drug behaviors in psychostimulant withdrawal (Taepavarapruk & Phillips, 2003; Barr *et al.*, 2014; Taepavarapruk *et al.*, 2014). Together, these findings suggest the hippocampal glucocorticoid system may play an important role in mediating stress hypersensitivity & relapse in amphetamine withdrawal. A greater understanding of this system could provide new targets for preventing stress-induced relapse. Therefore, the overall goal of these studies was to explore the effects of repeated amphetamine exposure on the ventral hippocampal glucocorticoid stress system in protracted withdrawal. These studies also aim to reveal whether the ventral hippocampal corticosterone system interacts with mesolimbic reward systems in control conditions and in protracted psychostimulant withdrawal (in rats).

The studies in chapter 2 were designed to test whether the corticosterone stress response is blunted in the ventral hippocampus during amphetamine withdrawal, which would explain the persistently abolished glucocorticoid-receptor-mediated serotonin stress response (Barr & Forster, 2011; Li *et al.*, 2014). Surprisingly, rats exposed to 20 min of restraint stress in the second week of withdrawal from repeated amphetamine exposure were found to have *enhanced* levels of free extracellular corticosterone in the ventral hippocampus relative to saline pre-treated controls (Chapter 2, Fig. 2-1). This suggests that the stress-induced serotonin deficit observed at the same withdrawal time point following the same amphetamine pre-treatment protocol (Li *et al.*, 2014) cannot be attributed to reduced glucocorticoid availability centrally.

In addition to glucocorticoid availability, a variety of possible mechanisms could contribute to the abolished glucocorticoid-receptor-mediated serotonin stress response in the ventral hippocampus during acute and protracted amphetamine withdrawal (Barr & Forster, 2011; Li *et al.*, 2014). Researchers from the Forster laboratory have investigated the likelihood of many of these possible mechanisms in either acute- or protracted psychostimulant withdrawal and many of the findings are summarized in Table A-2 and in Appendix 2.

Glucocorticoid receptor expression is reduced by ~30% in the ventral hippocampus during *acute* (< 24 h) amphetamine withdrawal (Barr & Forster, 2011). Expression and function of the low-affinity high-capacity organic cation transporter 3 (OCT-3, involved in high-level extracellular serotonin clearance) are also increased at this time point (expression by ~25%) (Barr *et al.*, 2013; Solanki *et al.*, 2016). These alterations may contribute to the acute serotonin deficiency (Barr & Forster, 2011). However, the OCT-3 alterations seem to be transient (Barr *et al.*, 2013; Solanki *et al.*, 2016) and corticosterone is known to inhibit OCT-3 (Grundemann *et al.*, 1998; Gasser, 2006; Gasser & Lowry, 2018). Therefore, the acute alterations in OCT-3 and glucocorticoid receptor expression (and OCT-3 function) do not seem to provide complete explanations for the complete and persistent inability of stress or ventral hippocampal glucocorticoid receptors to induce *any* local serotonin release in withdrawal (Barr & Forster, 2011; Li *et al.*, 2014).

It is possible that glucocorticoid receptor *function* or signaling is impaired in the ventral hippocampus during amphetamine withdrawal. This could occur in a way that prevents glucocorticoid receptor activation from recruiting serotonin signaling. The specific signaling

mechanisms by which glucocorticoid receptors induce serotonin release in the ventral hippocampus are unclear. It is possible that glucocorticoid receptors reside directly on serotonin terminals and induce terminal depolarization that stimulates serotonin release (Fig. 1-4D). It is also possible that glucocorticoid receptors reside on glutamatergic- or GABAergic interneurons that project onto local serotonin terminals in the ventral hippocampus (Fig. 1-4). Glucocorticoid receptors or their downstream signaling components could also interact directly with serotonin vesicles to induce vesicular release independent of terminal membrane depolarization. Potassium chloride depolarization has been shown to induce serotonin release in the ventral hippocampus in control conditions, but not in amphetamine withdrawal (Barr *et al.*, 2013). Therefore, it would be interesting to test whether blocking membrane depolarization by infusing a voltage-gated sodium channel blocker such as tetrodotoxin (TTX) (Consolo *et al.*, 1994) into the ventral hippocampus could prevent ventral hippocampal corticosterone infusions from stimulating extracellular serotonin release. Experiments to this end could help assess whether glucocorticoid receptors induce local serotonin release by directly stimulating terminal membrane depolarization. These findings could thus help clarify how glucocorticoid receptors induce some of their downstream effects.

Studies in the dorsal hippocampus and other limbic regions demonstrate that glucocorticoid receptors can recruit retrograde signaling mechanisms such as nitric oxide (NO, a gas) and 2-arachidonoylglycerol (2-AG, an ester/lipid) (Di *et al.*, 2003; Hu *et al.*, 2010; Di *et al.*, 2016, as addressed in chapters 1 and 3 and Fig. 1-4). This implies that the effects of glucocorticoid receptor activation can be diffuse and are not necessarily restricted by cell membranes. Therefore, the cellular localization of glucocorticoid receptors may not be relevant when

considering the possible mechanisms by which glucocorticoid receptor activation stimulates serotonin release. Rather, the cellular localization of glucocorticoid receptor signaling *responders* (NO-sensitive guanylyl cyclase (NO-GC) and inhibitory CB1 receptors) may be more important in determining the specific mechanisms by which glucocorticoid receptor activation can induce serotonin release locally.

It is unknown whether serotonin terminals in the ventral hippocampus express NO-GC. However, NO and nitric oxide synthase (NOS) *have* been demonstrated to mediate serotonin-induced behaviors (Bedrosian & Nelson, 2014; Ostadhadi *et al.*, 2015). If NO-GC is expressed in serotonin terminals in the ventral hippocampus (Fig. 1-4D), then activation of glucocorticoid receptors on any cell type in the ventral hippocampus could induce NO signaling that stimulates terminal serotonin release (through activation of NO-GC). Alternatively, inhibitory CB1 receptors are known to be located on GABAergic interneurons in the ventral hippocampus (Katona *et al.*, 1999). It is possible that interneurons expressing inhibitory CB1 receptors inhibit terminal serotonin release (Fig. 1-4D). This would suggest that glucocorticoid-receptor-mediated 2-AG synthesis (Di *et al.*, 2016) could initiate disinhibition of terminal serotonin output in the ventral hippocampus. This mechanism would occur regardless of where the glucocorticoid receptor itself is located.

In light of the above possibilities, it would be interesting to test whether the ability of stress or corticosterone in the ventral hippocampus to stimulate extracellular serotonin release (Barr & Forster, 2011; Li *et al.*, 2014) can be blocked by local infusions of NO synthesis (NOS) antagonists (such as N<sup>G</sup>-Nitro-L-arginine Methyl Ester, Hydrochloride) or by selective CB1

receptor antagonists (such as rimonabant or AM 251) (Di *et al.*, 2003; Beardsley *et al.*, 2009). The latter possibility would be particularly interesting since rimonabant and AM251 have been reported to block the induction of cocaine sensitization and reduce methamphetamine self-administration in rats (see review of literature in Beardsley *et al.*, 2009). Therefore, studies to this end could implicate NOS or endocannabinoids as possible pharmacotherapeutic interventions that may help correct the disrupted glucocorticoid signaling associated with anxiety and stress hypersensitivity in amphetamine withdrawal (Barr & Forster, 2011; Li *et al.*, 2014; Tu *et al.*, 2014).

Furthermore, the inability of glucocorticoid receptors to recruit serotonin signaling in the ventral hippocampus during amphetamine withdrawal (Barr & Forster, 2011; Li *et al.*, 2014) may have functional and behavioral effects that extend beyond the associated anxiety states (Tu *et al.*, 2014). For example, the loss of glucocorticoid-receptor-mediated serotonin signaling may contribute to the differential effects of ventral hippocampal corticosterone observed on accumbal dopamine output in control conditions and in amphetamine withdrawal (as addressed below). Also, a variety of literature suggests that glucocorticoid receptors may use serotonin signaling to regulate excitation and inhibition in the ventral hippocampus (Fig. 1-4D), as addressed in chapter 1 and in appendix 2 (section 1B) (Piguet & Galvan, 1994; Morales *et al.*, 1996; Freund & Gulyas, 1997; McMahon & Kauer, 1997; Liu *et al.*, 2000; Gruber *et al.*, 2015). This suggests that the loss of glucocorticoid-receptor-induced serotonin signaling in the ventral hippocampus during psychostimulant withdrawal could result in dysregulated inhibitory tone locally. Appendix 2 (section 1B) discusses the potential implications of this possibility. Uncovering the mechanisms that disrupt the ability of glucocorticoid receptors to recruit serotonin signaling

(Barr & Forster, 2011) can thus help provide basic information on the glucocorticoid system. Further exploring the possible effects of this disruption can help provide a better understanding of the neurobiological alterations that occur in the ventral hippocampus during amphetamine withdrawal.

Despite our finding of an enhanced corticosterone stress response in the ventral hippocampus in protracted amphetamine withdrawal (relative to that observed in saline pre-treated controls), the *plasma* corticosterone stress response did not differ from that observed in controls at the two time points tested (Chapter 2, Fig. 2-2). Total-, free-, and bound levels of plasma corticosterone were enhanced immediately after 20 minutes of restraint and reduced 1 hour after restraint (relative to stress-naïve controls), suggesting negative feedback regulation of the plasma corticosterone stress response in both control and withdrawal conditions, which is supported in the literature (van Haarst *et al.*, 1997; Russig *et al.*, 2006; Groeneweg *et al.*, 2011; Garrido *et al.*, 2012; Ergang *et al.*, 2014; Laryea *et al.*, 2014). However, the fact that no significant differences were observed in amphetamine withdrawal relative to control conditions suggests that the *enhanced* corticosterone stress response observed in the ventral hippocampus during protracted withdrawal cannot be explained by alterations in plasma corticosterone stress responses.

Further, the ratio of bound- to unbound plasma corticosterone levels did not differ as a function of time or pre-treatment (Chapter 2, Fig. 2-2). This suggests that the capacity for free corticosterone to reach the brain was equally unaffected by stress or amphetamine exposure and withdrawal immediately and 1 hour after restraint. Therefore, alterations in central access of peripheral corticosterone cannot explain the enhanced central corticosterone stress response at



these time points (Fig. 2-1). Our findings also suggest the enhanced central corticosterone stress response cannot be attributed to enhanced expression or function of the primary corticosteroidogenic enzyme in the brain, 11 $\beta$ -HSD1, or by enhanced expression of 11 $\beta$ -HSD1's cofactor, H6PD (however, see Appendix 1).

Like many other hormones, corticosterone has a binding protein, corticosterone binding globulin (CBG). CBG is thought to function as a buffer that can bind, store, and/or release free corticosterone to protect tissues from the deleterious effects of excessive or deficient corticosterone exposure (Moisan *et al.*, 2014). Interestingly, CBG can exist both peripherally *and centrally*, in the cerebrospinal fluid and in hippocampal cells (Schwarz & Pohl, 1992; Jirikowski *et al.*, 2007; Sivukhina *et al.*, 2013a; Sivukhina *et al.*, 2013b; Sivukhina & Jirikowski, 2014).

The microdialysis probes used to assess extracellular corticosterone levels in the ventral hippocampus in chapter 2 utilize membranes with 5 – 6 kDa MW cut-offs (Farmer *et al.*, 1996).

This membrane pore size enables passage of free corticosterone (0.35 kDa) through the membrane, but excludes bound corticosterone, since CBG is 50-60 kDa (Moisan *et al.*, 2014).

Therefore, it is possible that repeated amphetamine exposure and withdrawal alter synthesis, expression, or function of CBG centrally. This possibility could result in greater dissociation of bound corticosteroid in response to stress during amphetamine withdrawal. Greater dissociation of bound corticosterone centrally would then result in greater levels of free extracellular corticosterone (centrally) in response to stress, as was observed (Bray *et al.*, 2016; Chapter 2).

Therefore, altered central CBG binding or protein expression could explain the discrepancy between the enhanced central corticosterone stress response in withdrawal relative to the intact peripheral stress response (Chapter 2). Limitations in microdialysis membrane pore size prevent

accurate assessment of extracellular CBG protein levels centrally (as addressed in chapter 2).

Therefore, an initial future research directive could use immunostaining techniques (Jirikowski *et al.*, 2007) to assess whether CBG protein expression is altered in ventral hippocampal *tissue* during amphetamine withdrawal.

Overall, our findings suggest that repeated amphetamine exposure results in an enhanced corticosterone stress response in the ventral hippocampus during protracted withdrawal that is not accompanied by alterations to peripheral corticosterone stress responses (Bray *et al.*, 2016). However, the enhanced response may be associated with enhanced *behavioral* stress responses observed at the same time point following the same repeated amphetamine exposure protocol (Vuong *et al.*, 2010; Li *et al.*, 2014; Tu *et al.*, 2014). Thus, it is of importance to better understand the other possible functional effects of corticosterone in the ventral hippocampus as they relate to reward responses and behaviors, since establishing enhanced ventral hippocampal corticosterone as a direct cause of greater stress sensitivity in psychostimulant withdrawal could identify the glucocorticoid system as a novel target for treating behavioral symptoms of amphetamine withdrawal.

The studies in chapter 3 were designed to directly test whether stress-relevant concentrations of corticosterone in the ventral hippocampus can regulate dopamine reward systems, linking stress to incentive salience in control conditions and in protracted amphetamine withdrawal (Berridge & Robinson, 2016). We found that infusing stress-relevant concentrations of corticosterone into the ventral hippocampus *enhanced* accumbal dopamine output in control conditions (Chapter 3; Fig. 3-3A). Dopamine release in the nucleus accumbens shell is associated with incentive

salience, motivation, and the ability to suppress behavioral patterns that interfere with goal seeking (Floresco, 2014; Hollon *et al.*, 2015; Berridge & Robinson, 2016). Therefore, our findings suggest that the ventral hippocampal corticosterone stress response (Droste *et al.*, 2008; Bray *et al.*, 2016) may provide a mechanism that enables stress exposure to enhance reward salience and motivate goal-oriented behavior in normative states (Floresco, 2014; Hollon *et al.*, 2015; Berridge & Robinson, 2016). Furthermore, acute psychostimulant exposure is known to enhance corticosterone secretion (Knych & Eisenberg, 1979; Swerdlow *et al.*, 1993; Bayer *et al.*, 1995; Zuloaga *et al.*, 2014), which can also increase ventral hippocampal corticosterone levels in control and withdrawal conditions (Bray *et al.*, 2016). Therefore, our findings also suggest that corticosterone in the ventral hippocampus could provide a mechanism that contributes to positive reinforcement of initial psychostimulant use (Robinson & Berridge, 2000).

Interestingly, the same stress-relevant corticosterone infusions into the ventral hippocampus of amphetamine pre-treated rats rapidly *reduced* accumbal dopamine output during protracted amphetamine withdrawal (Chapter 3; Fig. 3-3A). Acute and persistent reductions in accumbal shell dopamine levels have been directly associated with aversive and dysphoric states that produce craving and drive drug-seeking and relapse (Shirayama & Chaki, 2006; Paliwal *et al.*, 2008; Wheeler *et al.*, 2008; Twining *et al.*, 2009; Wheeler & Carelli, 2009; Koob & Volkow, 2010; Gardner, 2011; Wheeler *et al.*, 2011; Twining *et al.*, 2014; Hurley *et al.*, 2017; Haake *et al.*, 2018). Therefore, our findings suggest that by reducing accumbal dopamine levels, the ventral hippocampal corticosterone stress-response (Bray *et al.*, 2016) may contribute to stress-induced craving and dysphoria that can prompt and negatively reinforce drug behaviors and relapse during periods of prolonged abstinence and withdrawal. However, whether corticosterone

in the ventral hippocampus *can* drive drug behaviors has not been directly tested. Therefore, it will be important for future work to clarify this, as addressed below.

Steroids (including corticosterone) can induce rapid effects that produce significant physiological alterations within less than 30 minutes of onset and are typically associated with non-genomic membrane-associated mechanisms (Makara & Haller, 2001; de Kloet *et al.*, 2008; Haller *et al.*, 2008; Prager & Johnson, 2009; Barr *et al.*, 2017). However, cytosolic and genomic mechanisms can also produce rapid effects on a similar timescale (Tumlin *et al.*, 1997; Croxtall *et al.*, 2002; Horvath & Wanner, 2006; Liu *et al.*, 2010; Scheschowitsch *et al.*, 2017; Mifsud & Reul, 2018). We found that the effects of corticosterone in the ventral hippocampus on accumbal dopamine release onset rapidly in control conditions and in amphetamine withdrawal (Chapter 3, sections 3.2a-b; Fig. 3-3A). However, the biphasic effect observed in amphetamine withdrawal (Chapter 3, section 3.2b; Fig. 3-3A) suggests the possibility of both non-genomic and genomic effects.

Furthermore, isolating corticosterone's effects to the cell membrane blocked the accumbal dopamine response (Chapter 3, section 4.4; Fig. 3-4) in saline pre-treated controls. This suggests that the excitatory effect observed in control conditions required cytosolic access. This finding does not preclude the underlying mechanisms from being non-genomic. Activation of cytosolic receptors and receptor complexes have been shown to induce non-genomic effects (Tumlin *et al.*, 1997; Croxtall *et al.*, 2000; Croxtall *et al.*, 2002; Liu *et al.*, 2010). Appendix 3 provides further discussion on support for this possibility. Furthermore, our findings do not exclude the involvement of membrane mechanisms. It is possible that cytosolic *and membrane* actions are *both* required (together) to mediate the observed excitatory effects of ventral hippocampal

corticosterone on accumbal dopamine release in control conditions (as addressed in chapter 3). These findings suggest the traditional differentiation of corticosterone's non-genomic mechanisms as being membrane-associated and cytosolic effects as being genomic may be oversimplified. Overall, our findings suggest the glucocorticoid system can induce both immediate and more long-term effects through similar or complimentary systems in healthy conditions and in disease states such as psychostimulant withdrawal (de Kloet *et al.*, 2008; Haller *et al.*, 2008).

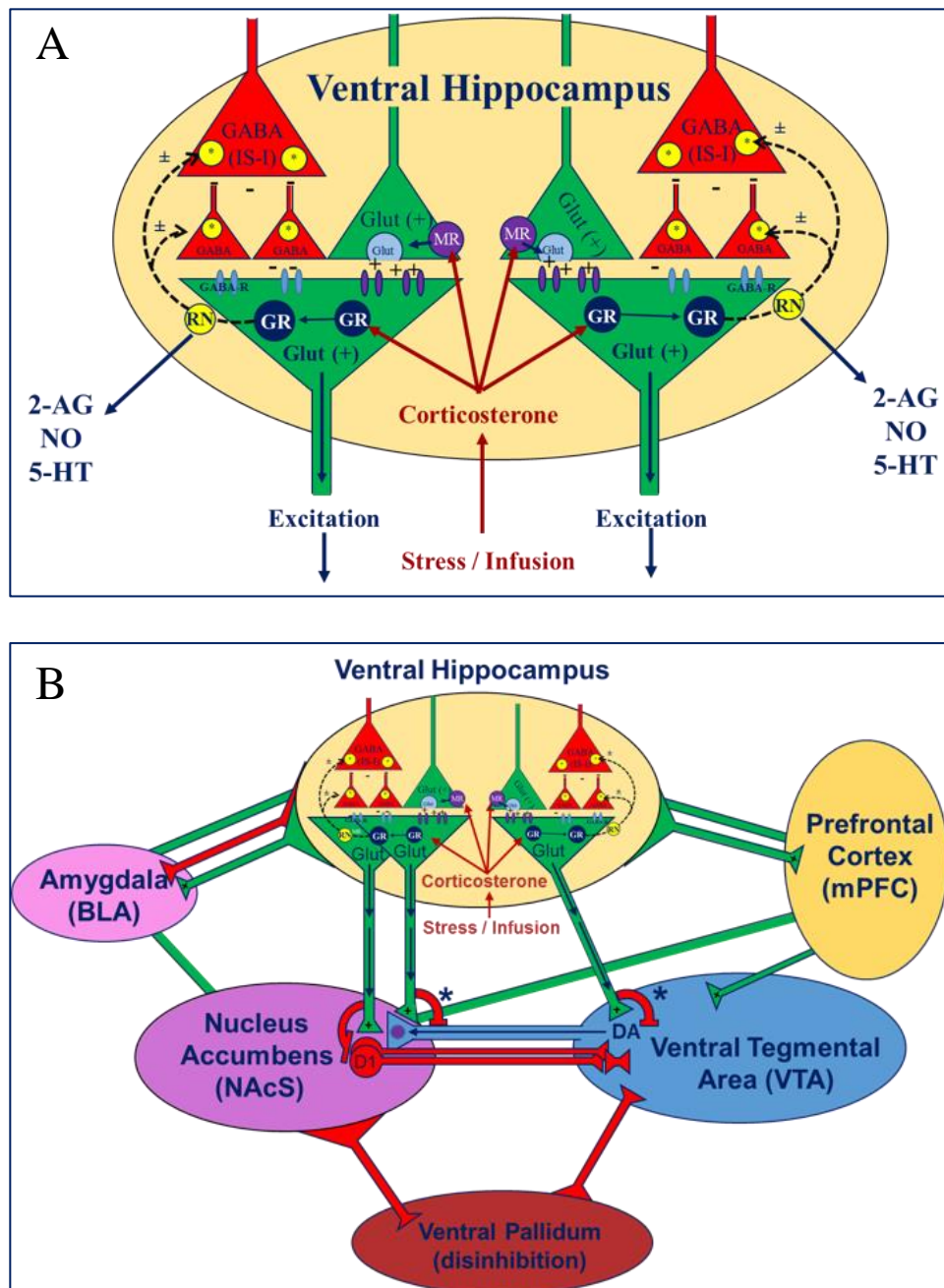
Studies in chapter 3 were also designed to explore the specific contributions of corticosterone's glucocorticoid and mineralocorticoid receptors to the differential effects of ventral hippocampal corticosterone on accumbal dopamine output. A variety of literature suggests that mineralocorticoid and glucocorticoid receptors in the ventral hippocampus can tightly regulate local excitation and inhibition through a variety of mechanisms that may critically involve the ability of glucocorticoid receptors to recruit serotonin signaling (as addressed in chapters 1 and 3). Therefore, the studies in chapter 3 were also designed to clarify whether the loss of glucocorticoid-receptor-mediated serotonin signaling (Barr & Forster, 2011; Li *et al.*, 2014) might be related to the differential dopamine responses observed in control conditions and in amphetamine withdrawal (3.2c in chapter 3; Fig. 3-3A). We selectively blocked corticosterone activation of either glucocorticoid or mineralocorticoid receptors in the ventral hippocampus. This was done by infusing selective doses of the respective receptor antagonists into the ventral hippocampus prior to corticosterone infusions (Heikinheimo, 1997; Mahajan & London, 1997; Garthwaite & McMahon, 2004).

We found that selectively blocking either mineralocorticoid or glucocorticoid receptors in the ventral hippocampus prevented hippocampal corticosterone from altering accumbal dopamine output in both control conditions and in amphetamine withdrawal (Chapter 3, section 3.2d; Figs. 3-3B and 3-3C). Blocking either receptor revealed a nullified effect of the other receptor when independently activated. These findings suggest that neither glucocorticoid- nor mineralocorticoid receptors in the ventral hippocampus are sufficient to independently enable ventral hippocampal corticosterone to alter accumbal dopamine output. Furthermore, independently blocking *either* receptor subtype was found to block corticosterone's actions in both controls and withdrawal conditions. This suggest that although *neither* receptor subtype can be *independently* attributed with enabling corticosterone to alter accumbal dopamine release, *both* receptor subtypes seem to be *required* to produce the observed effects.

At the genomic level, a broad variety of literature suggests that concomitant activation of mineralocorticoid and glucocorticoid receptors may be required for activation of specific glucocorticoid target genes (Mifsud & Reul, 2016; Mifsud & Reul, 2018). This has been proposed to occur through heterodimerization of mineralocorticoid and glucocorticoid complexes (MR:GR heterodimerization). Importantly, MR:GR heterodimerization is thought to require concomitant activation of both mineralocorticoid and glucocorticoid receptors. Moreover, the downstream effects differ from those that can be elicited by independent activation of mineralocorticoid or glucocorticoid receptors or homodimers (Liu *et al.*, 1995; Trapp & Holsboer, 1996; Ou *et al.*, 2001; Mifsud & Reul, 2016; Mifsud & Reul, 2018). This cooperative ability increases the functional diversity of corticosterone's actions at the genomic level (Trapp & Holsboer, 1996). Furthermore, *genomic* MR:GR heterodimerization has been

demonstrated both *in vitro* and *in vivo*, and in ventral hippocampal tissue. Therefore, we propose that corticosterone's *non-genomic* receptors may *also* possess the capacity to induce distinct *non-genomic* effects that can only be elicited by activation of *both* glucocorticoid and mineralocorticoid receptors, as has been observed in corticosterone's *genomic* receptors. Appendix 3 provides an additional discussion on further support for this proposal.

Based on our findings in chapter 3 and on existing literature about mineralocorticoid and glucocorticoid receptors, we conclude that ventral hippocampal corticosterone alters accumbal dopamine output through compulsory cooperative activation of membrane mineralocorticoid receptors and cytosolic glucocorticoid receptors in both control conditions and in protracted amphetamine withdrawal (section 4.6 in chapter 3 and Fig. 4-1A). Our hypothesis that corticosterone's rapid effects on accumbal dopamine output require activation of excitatory non-genomic membrane mineralocorticoid receptors are based on findings that these receptors can activate voltage-gated calcium channels to increase excitatory postsynaptic potential (EPSP) slope in the ventral hippocampus (Maggio & Segal, 2007) and potentiate glutamatergic transmission in CA1 tissues (Karst *et al.*, 2005) *in vitro*. However, non-genomic mineralocorticoid receptors in the ventral hippocampus have also been found to reduce the frequency of spontaneous inhibitory post-synaptic currents (sIPSCs) (Maggio & Segal, 2009), suggesting they can also reduce the probability of extracellular *GABA* release and may additionally have disinhibiting properties (as acknowledged in Fig. 1-4D).



**Figure 4-1. Mechanisms proposed to enable ventral hippocampal corticosterone to differentially alter accumbal dopamine output in control conditions and following repeated amphetamine exposure and withdrawal. A) Cellular/molecular mechanisms proposed to mediate corticosterone excitation within the ventral hippocampus.** At the cellular/molecular level within the ventral hippocampus, we propose that glutamatergic efferents from the ventral hippocampus (shown in green) may be under tonic inhibition by GABAergic interneuron- and interneuron-specific (IS) interneurons (shown in red). We conclude that corticosterone activation of presynaptic membrane mineralocorticoid receptors (MR, purple circles) potentiates local glutamate release onto the ventral hippocampus' glutamatergic efferents. However, activation of postsynaptic cytosolic glucocorticoid receptors (GR, orange circles) is also required to induce top-down disinhibition of the glutamatergic efferents (as indicated by dashed arrows), whose terminal actions regulate accumbal dopamine output. The disinhibiting effects of GR activation are thought to occur through its induction of 2-Arachidonoylglycerol (2-AG), nitric oxide (NO), and/or serotonin (5-HT) signaling which have been shown to act on inhibitory G<sub>i/o</sub>-coupled Type I cannabinoid (CB1) receptors, NO-sensitive guanylyl cyclase, and serotonin's inhibitory G<sub>i</sub>-coupled Type 1A (5-HT<sub>1A</sub>) and excitatory ligand-gated cation channel Type III (5-HT<sub>3</sub>) receptors (respectively) on the presynaptic terminals of presynaptic GABAergic interneurons. We propose this ultimately disinhibits



*GABAergic suppression of the ventral hippocampus' principal glutamatergic efferents that project to the mesolimbic dopamine system, enabling depolarizing events that regulate accumbal dopamine output. B) Neural circuits proposed to mediate the different effects of ventral hippocampal corticosterone on accumbal dopamine output in control conditions and in amphetamine withdrawal. The disynaptic terminals of the vHipp projections onto NAcS MSNs are thought to regulate the excitation/inhibition (e/i) balance within the NAcS, ultimately inducing greater MSN inhibition, which is disrupted following repeated cocaine exposure (Scudder, 2018). It is unknown whether other glutamatergic efferents from the ventral hippocampus – such as those projecting onto VTA-NAcS DA projections – also exhibit disynaptic terminals. Amphetamine withdrawal is known to alter synaptic connections in the NAc (Scofield et al., 2016); thus, we propose that repeated amphetamine exposure and withdrawal enhances inhibitory tone in the NAcS or VTA and disrupts overall midbrain e/i balance such that excitatory output from the ventral hippocampus has an inhibitory and reducing effect on accumbal dopamine release in withdrawal. 2-AG: 2-Arachidonoylglycerol; 5-HT: Serotonin; BLA: Basolateral amygdala; D1: Type-1 dopamine receptor-expressing medium spiny neuron; DA: Dopamine; Glut: Glutamate; GR: Glucocorticoid receptor; IS-I: Interneuron-specific interneuron; mPFC: Medial prefrontal cortex; MR: membrane mineralocorticoid receptor; NAcS: Nucleus accumbens shell; NO: Nitric Oxide; RN: Retrograde neurotransmitter; VTA: ventral tegmental area. Green projections represent principal glutamatergic projections; red projections represent GABAergic projection neurons and interneurons.*

Our hypothesis that corticosterone's rapid effects on accumbal dopamine output also require activation of disinhibitory non-genomic glucocorticoid receptors *in the cytosol* is based on several findings. Cytosolic glucocorticoid receptors can rapidly and reversibly inhibit ATP-induced  $\text{Ca}^{2+}$  influxes in rat dorsal root ganglion cultures (Liu *et al.*, 2010). This inhibitory effect was blocked with mifepristone and could not be reproduced with membrane impermeable corticosterone (Liu *et al.*, 2010). These findings mirror our findings that accumbal dopamine responses were blocked with mifepristone and membrane-impermeable corticosterone (sections 3.2d and 3.3 of chapter 3). *In vitro* studies in *ventral hippocampal* tissue also demonstrate that glucocorticoid receptors can increase the magnitude of inhibitory postsynaptic currents (IPSCs) (Maggio & Segal, 2009). This effect was found to onset slowly with large potentiation peaking within 45 – 60 min after perfusing hippocampal slices with the glucocorticoid receptor agonist dexamethasone, and was also blocked by mifepristone (Maggio & Segal, 2009). This finding aligns temporally with our findings that corticosterone infusions into the ventral hippocampus produce alterations in accumbal dopamine output that peak 57.5 min post-infusion in control conditions and 67.5 min post-infusion (2<sup>nd</sup> peak) in amphetamine withdrawal, and are blocked by mifepristone in both conditions (Chapter 3, section 3.2; Fig. 3-3).

The presence of local interneurons, interneuron-*inhibiting* interneurons (IS-Is) and long-range GABAergic projections in the hippocampus (Figs. 1-4D, 4-1) (Freund & Buzsaki, 1996; Jinno, 2009; Chamberland & Topolnik, 2012) make it difficult to discern whether the glucocorticoid-receptor-mediated effects on  $\text{Ca}^{2+}$  influxes and IPSCs discussed above (Maggio & Segal, 2009; Liu *et al.*, 2010) would translate to inhibitory or disinhibitory effects locally and/or in the nucleus accumbens shell *in vivo*. This is because it is not known whether these glucocorticoid-receptor effects are mediated by direct activation of glucocorticoid receptors located in interneurons or IS-Is. It is also not known whether the effects are mediated by glucocorticoid receptor recruitment of diffuse retrograde signaling mechanisms which could induce the effects in (and from) virtually any cell type locally. The ability of glucocorticoid receptors to recruit serotonin release presents a similar confound. As addressed above and in chapter 1, serotonin's excitatory and inhibitory receptors can be located on virtually all cell types in the ventral hippocampus and excitatory 5-HT<sub>3</sub> receptors seem to be preferentially expressed on interneurons and IS-Is (see above for relevant citations). Moreover, it is not known whether glucocorticoid receptors recruit extracellular serotonin release through their direct actions on serotonin terminals, excitatory or inhibitory terminals that influence serotonin output, or through their recruitment of retrograde neurotransmission. Overall, future research can benefit from clarifying the downstream signaling mechanisms implored by glucocorticoid and mineralocorticoid receptors in the ventral hippocampus *in vivo* and the effects of each receptor subtype on extracellular levels of glutamate and GABA in the ventral hippocampus in control conditions and during acute and protracted psychostimulant withdrawal (as addressed above, and below).

It would also be beneficial to test whether *serotonin* infusions into the ventral hippocampus can alter accumbal dopamine output in control conditions and in acute and protracted psychostimulant withdrawal. Clarifying this question could shed light on whether the loss of glucocorticoid-receptor-mediated serotonin signaling observed in acute and protracted withdrawal (Barr & Forster, 2011; Li *et al.*, 2014) contributes to the differential effects of ventral hippocampal corticosterone on accumbal dopamine output (Fig. 3-3A). Clarifying this question could also potentially implicate the deficient serotonin response (Barr & Forster, 2011; Li *et al.*, 2014) in contributing to the states of dysphoria that result from accumbal dopamine deficiency and that drive drug behaviors (Gardner, 2011; Twining *et al.*, 2014).

Chapter 3 introduces recent findings that a population of disynaptic glutamatergic efferents from the ventral hippocampus to the medial nucleus accumbens shell shift the local excitation/inhibition balance from a “backdrop of inhibition” to an excitatory state in acute cocaine withdrawal (Scudder *et al.*, 2018; Figs. 1-3 and 4-1B). We propose additional plastic changes in these disynaptic connections may shift the excitatory/inhibitory balance (Scudder *et al.*, 2018) to one favoring inhibition in protracted withdrawal (Fig. 4-1B). The observed shift could result in excitatory output from the ventral hippocampus to the nucleus accumbens shell having an excitatory effect on accumbal dopamine release in control conditions and a *reducing* effect in protracted withdrawal (as we observed in chapter 3, Fig. 3-3A). It is unknown whether the ventral hippocampus’ other glutamatergic efferents (such as those to the ventral tegmental area) are also disynaptic (Fig. 4-1B). It is also unknown whether these disynaptic efferents actually *do* undergo plastic changes in their connectivity during protracted amphetamine withdrawal. Therefore, it would be interesting to test whether selectively blocking either D<sub>1</sub> or

D<sub>2</sub> receptors in the nucleus accumbens shell can differentially alter the ability of ventral hippocampal corticosterone to influence accumbal shell dopamine release in control conditions and during acute and protracted amphetamine withdrawal. This could be tested by infusion either the D<sub>1</sub> antagonist SCH-23,390 or SKF-83,959 (Zhang *et al.*, 2009) or the D<sub>2</sub> antagonist quinprole (Weber *et al.*, 2018) into the nucleus accumbens shell prior to infusing corticosterone into the ventral hippocampus with accumbal dopamine output measured by *in vivo* chronoamperometry (as per chapter 3).

#### *4.1. Additional Future Research Directives*

Overall, future research can benefit from clarifying the downstream signaling mechanisms implored by glucocorticoid and mineralocorticoid receptors in the ventral hippocampus *in vivo* and the effects of each receptor subtype on extracellular levels of *glutamate and GABA* in the ventral hippocampus in control conditions and during acute and protracted psychostimulant withdrawal. It would further be of benefit to clarify the receptor mechanisms that glucocorticoid receptors utilize to contribute to the ability of ventral hippocampal corticosterone to alter accumbal dopamine output in control conditions and in acute and protracted psychostimulant withdrawal, and clarify the specific effects of extracellular serotonin release in the ventral hippocampus on accumbal dopamine output (in control conditions and in psychostimulant withdrawal *in vivo*). Such research could shed light on whether repeated psychostimulant exposure alters local excitatory/inhibitory tone in the ventral hippocampus during acute and protracted periods of withdrawal, and whether the reduced glucocorticoid receptor expression and dysregulated glucocorticoid/mineralocorticoid receptor ratio in acute (< 24 h) withdrawal (Barr & Forster, 2011) – or the persistent loss of glucocorticoid-receptor-mediated serotonin

signaling in acute and protracted withdrawal (< 24 h and 2 weeks) (Barr & Forster, 2011; Li *et al.*, 2014) – influence the excitation/inhibition balance in the ventral hippocampus (and resulting effects on its downstream targets). It will also be important to test whether corticosterone in the ventral hippocampus *enhances* accumbal dopamine excitation during acute (< 24 h ) psychostimulant withdrawal, similar to the effects observed following ventral hippocampal NMDA infusions at that same time point (Barr *et al.*, 2014).

Finally, it will be of paramount importance for future research to test whether corticosterone in the ventral hippocampus *can* directly drive drug behaviors during periods of acquisition as well as during acute and protracted periods of psychostimulant withdrawal. This line of work would directly link the ventral hippocampal corticosterone system to drug taking and relapse. It would be fruitful to pair *in vivo* microdialysis and high-pressure liquid chromatography (HPLC) with self-administration techniques. A rat model of amphetamine self-administration and extinction could be used to test whether stress-relevant infusions of corticosterone in the ventral hippocampus (Barr & Forster, 2011) can increase frequency or amount of psychostimulant self-administration during the acquisition phase and immediately after extinction (Taepavarapruk *et al.*, 2014; Miszkiel *et al.*, 2018). Freely moving microdialysis and HPLC techniques could be used to test whether the same stress-relevant infusions of corticosterone in the ventral hippocampus (Barr & Forster, 2011) can also differentially alter extracellular levels of glutamate or GABA (in the ventral hippocampus) (Venero & Borrell, 1999; Zhang *et al.*, 2005) in saline and amphetamine pre-treated rats. These studies could link the ventral hippocampal corticosterone stress response with the known ability of stress and aversive stimuli to enhance and reinstate drug-taking behaviors (Sinha *et al.*, 1999; Sinha *et al.*, 2000; Sinha, 2001; Weise-

Kelly & Siegel, 2001; Sinha *et al.*, 2003; Ungless *et al.*, 2004; Sinha *et al.*, 2006; Sinha, 2007; Paliwal *et al.*, 2008; Roitman *et al.*, 2008; Wheeler *et al.*, 2008; Brischoux *et al.*, 2009; Twining *et al.*, 2009; Wheeler & Carelli, 2009; Wheeler *et al.*, 2011; Robinson *et al.*, 2014; Twining *et al.*, 2014; Hurley *et al.*, 2017; Haake *et al.*, 2018) at different stages within the addiction cycle (Gardner, 2011; Koob *et al.*, 2014; Kwako & Koob, 2017). These studies could clarify whether corticosterone in the ventral hippocampus can alter local glutamate and GABA release *in vivo*. These studies could be followed by testing whether infusions of mifepristone or spironolactone (or antagonists for known mineralocorticoid and glucocorticoid receptor signaling components, such as the NOS antagonists N<sup>G</sup>-Nitro-L-arginine Methyl Ester, Hydrochloride (Di *et al.*, 2003), the selective CB1 receptor antagonists rimonabant or AM 251 (Beardsley *et al.*, 2009) or the 5-HT<sub>3</sub> antagonist tropisetron (Dorostkar & Boehm, 2007)) can alter the effects of ventral hippocampal corticosterone on extracellular levels of glutamate and GABA locally and on drug behaviors. These studies could strongly link the corticosterone stress response in the ventral hippocampus (Bray *et al.*, 2016) to relevant drug behaviors. The antagonist studies could also help identify novel pharmacotherapeutic targets that can offer protection from the deleterious effects of stress on relapse in withdrawal.

## **Overall Conclusions**

The overall goal of this research is to explore the neurobiological alterations that occur in the ventral hippocampal corticosterone stress system in response to repeated psychostimulant exposure and withdrawal. In a face-valid rat model of amphetamine pre-treatment and withdrawal, we found that the corticosterone stress response is *enhanced* in the ventral hippocampus during protracted amphetamine withdrawal. Corticosterone is thought to be

excitatory in the ventral hippocampus and excitation in the ventral hippocampus can stimulate accumbal shell dopamine output and directly drive drug behaviors. Here, we demonstrated for the first time that stress-relevant concentrations of corticosterone infused into the ventral hippocampus of anesthetized rats rapidly *enhance* accumbal shell dopamine release in control conditions but produce a biphasic reduction in dopamine output during amphetamine withdrawal. This suggests that corticosterone in the ventral hippocampus may contribute to the differential effects of stress on accumbal dopamine release and behavioral outcomes in control conditions and in drug withdrawal. Separately blocking corticosterone activation of mineralocorticoid-, glucocorticoid-, or cytosolic receptors prevented the effects. Therefore, we conclude that corticosterone in the ventral hippocampus alters accumbal dopamine release through obligatory activation of disinhibitory cytosolic glucocorticoid receptors and excitatory membrane mineralocorticoid receptors. Overall, our findings reveal a *bona fide* role for corticosterone in the ventral hippocampus as contributing to the transition from positively reinforced drug taking to negatively reinforced drug dependence that maintains the addiction cycle. Uncovering treatments that can prevent the ability of ventral hippocampal corticosterone to reduce accumbal dopamine release may help prevent stress-induced relapse in withdrawal.

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## Appendix 1: Assessing 11 $\beta$ -HSD1 expression and activity of in *ex vivo* homogenates

### Cautionary notes

In chapter 2, we failed to detect an effect of stress or amphetamine exposure and withdrawal on functional expression or activity of 11 $\beta$ -HSD1 *protein* in *ex vivo* homogenized tissue samples obtained from the *entire* ventral hippocampus (2.2.2-3, Figs. 2-3A & 2-4A; Bray *et al.*, 2016). In contrast, a variable 3-day stress protocol was found to increase 11 $\beta$ -HSD1 *mRNA* expression in the ventral (but not dorsal) CA1 region of the hippocampus (Ergang *et al.*, 2014) and 7 days of exposure to a resident-intruder paradigm of social stress significantly increased 11 $\beta$ -HSD1 *mRNA* expression in the ventral (but not dorsal) CA1 (but not CA3) region of the hippocampus in both residents and intruders, with intruders also showing increased 11 $\beta$ -HSD1 *mRNA* expression in the CA2 region (Vodicka *et al.*, 2014). The regional specificity of these findings suggests that although we did not observe significant alterations in 11 $\beta$ -HSD1 *protein* expression or enzyme activity in tissue obtained from the *entire* ventral hippocampus, it is possible that repeated amphetamine exposure or stress *do* increase 11 $\beta$ -HSD1 protein expression or enzyme activity in the CA1 region specifically in a way that was confounded by our inclusion of CA2 and CA3 tissues.

Further, our *ex vivo* findings must be interpreted with caution, as 11 $\beta$ -HSD1 activity *in vivo* critically depends on the cellular microenvironment (Seckl, 1997), which is disrupted *ex vivo* by oxidative processes (Seckl, 1997; Banhegyi *et al.*, 2008; Dixon *et al.*, 2008) and homogenation (Moisan *et al.*, 1990; Lakshmi *et al.*, 1991; Seckl, 1997). For example, the directional activity of 11 $\beta$ -HSD1 is driven by H6PD activity, which is driven in turn by H6PD reduction of NADP<sup>+</sup> to

NADPH and by H6PD oxidation of glucose 6-phosphoate (G6P) to 6-phosphogluconase (6PG) (as part of the pentose phosphate pathway) (Hewitt *et al.*, 2005; Czege *et al.*, 2012; Chapman *et al.*, 2013; Legeza *et al.*, 2013). Others have found that *ex vivo* oxidative processes and homogenation can disrupt the ratio of NADPH/NADP<sup>+</sup> (Moisan *et al.*, 1990; Lakshmi *et al.*, 1991; Seckl, 1997; Banhegyi *et al.*, 2008). Disrupted ratios of NADPH/NADP<sup>+</sup> could disrupt *ex vivo* activity of H6PD and 11 $\beta$ -HSD1 in turn, thus confounding our *ex vivo* activity assay findings. Similarly, 11 $\beta$ -HSD1 resides in the ER lumen in peripheral tissues (Czege *et al.*, 2012; Legeza *et al.*, 2013) and acute stress exposure has been found to disrupt ER redox status in a variety of neurological diseases (Banhegyi *et al.*, 2008), which suggests the possibility for acute stress during psychostimulant withdrawal to transiently disrupt redox status – and 11 $\beta$ -HSD1 activity or directionality (and subsequent corticosteroid availability) in the ventral hippocampus – *in vivo* in a way that cannot be detected *ex vivo*. Therefore, future research endeavors aimed at investigating the possible effects of central 11 $\beta$ -HSD1 activity on corticosteroidogenesis should do so *in vitro* or *in vivo*, but not *ex vivo*.

It is worth noting that 11 $\beta$ -HSD2 acts as a dehydrogenase to inactivate glucocorticoids in central tissue during fetal development (Wyrwoll *et al.*, 2011) but is not present in adult rat hippocampal tissue (Roland *et al.*, 1995; Wyrwoll *et al.*, 2011; Odermatt & Kratschmar, 2012), and preliminary western immunoblotting assay in our lab (not shown) also did not identify 11 $\beta$ -HSD2 expression in ventral hippocampal tissue of adult male Sprague Dawley rats.

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## **Appendix 2: Neurobiological alterations in the ventral hippocampus during acute and protracted psychostimulant withdrawal.**

Studies from our laboratory suggest that acute and protracted amphetamine withdrawal may represent two discrete time periods within the addiction cycle that can be linked to distinct neurobiological alterations in the ventral hippocampal glucocorticoid system. However, there remain many gaps in the literature regarding specific alterations that occur in acute and protracted withdrawal (Table A-2). Filling in these gaps could reveal a clearer picture of what neurobiological alterations observed in acute withdrawal persist into protracted withdrawal periods, and what dysregulations observed in protracted withdrawal are also present acutely. Filling in these gaps can also clarify what neurobiological alterations mediate stress hypersensitivity in withdrawal, how the ventral hippocampal glucocorticoid system changes throughout the withdrawal prognosis, and what the functional and behavioral effects of these changes are.

### **1. Acute (< 24 h) psychostimulant withdrawal: Local serotonin deficit; possible disinhibition in the ventral hippocampus; enhanced excitatory output to the nucleus accumbens shell; shift toward excitation in the accumbens shell**

#### *A. Local Serotonin Deficit*

Acute psychostimulant withdrawal (< 24 h) represents an emergent serotonin deficit marked by reduced basal levels of serotonin in the ventral hippocampus that are not present 4 weeks later (Barr *et al.*, 2010). Reduced expression of serotonin clearance mechanisms seem to be transient and specific to this acute time point (Solanki *et al.*, 2016); whereas suppressed stress-induced

serotonin responses seem to persist 2 weeks out (Barr & Forster, 2011; Li *et al.*, 2014). The serotonin deficit may result from reduced serotonin input from the raphe nucleus, though this should be confirmed by future research.

#### *B. Possible Disinhibition in the Ventral Hippocampus and Enhanced Excitatory Output/Drive*

The acute withdrawal time point is also marked by reduced glucocorticoid receptor expression in the ventral hippocampus and a loss of glucocorticoid-receptor-mediated serotonin signaling (Barr & Forster, 2011) and enhanced excitatory drive from the ventral hippocampus to the nucleus accumbens shell (Barr *et al.*, 2014). The increased excitatory drive from the ventral hippocampus to the nucleus accumbens shell (Barr *et al.*, 2014) may result from reduced inhibitory tone within the ventral hippocampus during acute withdrawal. Although it is not currently known whether inhibitory tone *is* reduced in the ventral hippocampus during acute withdrawal, it *is* known that activation of glucocorticoid receptors in the ventral hippocampus can increase the magnitude of inhibitory postsynaptic currents (IPSCs) *in vitro* (Maggio & Segal, 2009), which may occur through serotonin signaling, as glucocorticoid receptors are known to induce serotonin release in the ventral hippocampus in control conditions *in vivo* (but not in acute amphetamine withdrawal) (Barr & Forster, 2011; Li *et al.*, 2014). Although serotonin's excitatory and inhibitory receptors can be located on nearly all cell types within the hippocampus (Berumen *et al.*, 2012; Bombardi, 2012; Tanaka *et al.*, 2012; Pehrson *et al.*, 2016), excitatory 5-HT<sub>3</sub> receptor expression seems to be confined to interneurons (of almost all varieties), including interneuron-specific interneurons (IS-Is) (Freund & Buzsaki, 1996; Berumen *et al.*, 2012; Chamberland & Topolnik, 2012; Pelkey *et al.*, 2017) and cholecystokinin expressing (CCK+) interneurons (Morales *et al.*, 1996; Pelkey *et al.*, 2017). Together, these findings would suggest that the loss of glucocorticoid-receptor-

induced serotonin signaling in the ventral hippocampus during acute psychostimulant withdrawal could result in reduced inhibitory tone locally at this time point.

The fine-tuned regulation of hippocampal excitation through highly sophisticated interneuronal networks is highly adaptive and is thought to help prevent excessive excitation within the hippocampus (Freund & Buzsaki, 1996). This could serve to buffer the effects of small excitatory micro-fluctuations within the ventral hippocampus from negatively impacting the ventral hippocampus' efferent targets (though to my knowledge there is no current literature investigating this). Therefore, a reduced inhibitory tone locally during acute (and protracted) psychostimulant withdrawal could result in disinhibited stress responses that could ultimately translate to enhanced behavioral responses to stress in acute psychostimulant withdrawal. This could also explain why Barr et al., 2014 found that NMDA infusions were sufficient to excite accumbal dopamine output in acute (<24 h) withdrawal from repeated cocaine exposure; whereas we propose that glucocorticoid-receptor-mediated disinhibition of the ventral hippocampus' glutamatergic efferents to the nucleus accumbens shell is required in order for *corticosterone* in the ventral hippocampus to induce its effects on accumbal dopamine release in control conditions and in *protracted* (2 weeks) amphetamine withdrawal (chapter 3).

### *C. Enhanced Excitatory Drive: Behavioral Implications*

It will be important for future research to confirm whether local inhibitory tone in the ventral hippocampus *is* altered during acute or protracted psychostimulant withdrawal, and whether this results in enhanced excitatory drive from the ventral hippocampus to its downstream targets (Barr *et al.*, 2014). Enhanced excitatory output from the ventral hippocampus could result in

greater ventral hippocampus-mediated interoceptive drive (Barr *et al.*, 2017) to the nucleus accumbens shell during acute psychostimulant withdrawal, which could enhance tolerance (Kim *et al.*, 1999; Weise-Kelly & Siegel, 2001; Twining *et al.*, 2009) and drive drug behaviors that can accelerate drug dependence and crystalize addiction (Sinha, 2007; Cleck & Blendy, 2008; Paliwal *et al.*, 2008; Wheeler *et al.*, 2008; Twining *et al.*, 2009; Wheeler & Carelli, 2009; Wheeler *et al.*, 2011; Twining *et al.*, 2014). Therefore, it would be beneficial for future studies test this possibility in relevant behavioral models of self-administration, extinction, and stress-induced reinstatement, (as discussed in chapter 4).

## **2. Protracted psychostimulant withdrawal (2 weeks): Enhanced corticosterone stress responses in the ventral hippocampus; possible enhanced inhibitory tone in the ventral hippocampus and mesolimbic system**

### *A. Enhanced Corticosterone Stress Response in the Ventral Hippocampus*

Protracted amphetamine withdrawal (2 weeks) seems to represent a separate, distinct time period, characterized by an enhanced corticosterone stress response in the ventral hippocampus that is not mediated by alterations to peripheral corticosterone stress responses or primary corticosteroidogenic enzymes (Bray *et al.*, 2016), but which may instead result from blood brain barrier damage (which is proposed to enable central access to bound corticosterone from the periphery) and subsequent alterations to corticosterone binding mechanisms and dynamics centrally (see discussion in chapter 3 for relevant citations). It will be important to test this possibility going forward, as understanding the mechanisms that mediate the enhanced central corticosterone stress response during protracted amphetamine withdrawal could reveal new pharmacotherapeutic treatment targets that can normalize the central- and behavioral stress

hyper-responsivity observed in protracted psychostimulant withdrawal (Li *et al.*, 2014; Bray *et al.*, 2016), thus helping to prevent stress-induced relapse that can maintain the addiction cycle (Sinha, 2007; Paliwal *et al.*, 2008).

#### *B. Persistent Disruption of Stress-Induced Serotonin Release in the Ventral Hippocampus*

While it is unknown whether basal levels of serotonin remain reduced in the ventral hippocampus during the second week of psychostimulant withdrawal (Barr *et al.*, 2010), serotonin clearance mechanisms seem to be normalized at this time point (Solanki *et al.*, 2016), yet the deficient stress-induced serotonin response persists (Li *et al.*, 2014). This may result from a deficit in serotonergic supply from the raphe nucleus, since it seems unlikely that glucocorticoid receptor expression is reduced at this time point (so the serotonin deficiency cannot likely be attributed to reduced glucocorticoid receptor expression, as addressed in chapter 4). Again, it will be beneficial for future research to test whether serotonin output from the raphe nucleus to the ventral hippocampus *is* altered in acute and protracted withdrawal, since the serotonin deficit in the ventral hippocampus is directly linked to anxiety states (Tu *et al.*, 2014) that are associated with relapse during withdrawal (Hellem, 2016; Hartel-Petri *et al.*, 2017). Thus, understanding the underlying mechanisms may help uncover novel treatment targets that can be exploited to prevent the serotonin deficit that drives anxiety and may be also linked to stress hypersensitivity in acute and protracted amphetamine withdrawal.

#### *C. Alterations in Mesolimbic Inhibition/Excitation Proposed to Explain the Differential Effects of Ventral Hippocampal Corticosterone and Excitation on Accumbal Dopamine Output in Control Conditions and in Acute and Protracted Psychostimulant Withdrawal*

Ventral hippocampal corticosterone *enhances* accumbal shell dopamine output in control conditions; whereas it has the opposite effect and *reduces* accumbal shell dopamine output in protracted amphetamine withdrawal (chapter 3, Fig. 3-3A). As addressed above, the effects of ventral hippocampal corticosterone on accumbal dopamine output during acute (<24 h) psychostimulant withdrawal are unknown. However, we propose that corticosterone in the ventral hippocampus – which we presume to have an excitatory effect on local glutamate release – would *enhance* accumbal shell dopamine excitation relative to control levels during acute psychostimulant withdrawal as a result of the proposed reduction in local inhibitory tone within the ventral hippocampus during acute (< 24 h) psychostimulant withdrawal. We propose that the reducing effect of ventral hippocampal corticosterone on accumbal dopamine output observed in *protracted* withdrawal may result from enhanced inhibitory tone *within the mesolimbic system* during this time point (see discussion in chapters 3 and 4 for relevant citations). Thus, we conclude that plastic alterations in excitation/inhibition both within the ventral hippocampus and within the mesolimbic system may result in excitatory output from the ventral hippocampus having an excitatory effect on accumbal dopamine output in control conditions (Barr *et al.*, 2014), an *enhanced* excitatory effect during acute psychostimulant withdrawal (Barr *et al.*, 2014) (which we propose to be mediated by a shift toward excitation within the nucleus accumbens shell, as reported by Scudder *et al.*, 2018), but a *reducing* effect in protracted amphetamine withdrawal.

Specifically, we theorize that the ventral hippocampus' disynaptic projections responsible for mediating excitation/inhibition balance in the nucleus accumbens shell – which have been found to favor inhibition in control conditions but shift to excitation in *acute* psychostimulant

withdrawal (Scudder *et al.*, 2018) – may further shift to favor inhibition more strongly during *protracted* psychostimulant withdrawal in a way that is relevant not only to the “inhibitory backdrop” of medium spiny neurons in the nucleus accumbens shell (as Scudder *et al.* propose) but also in a way that is relevant to dopamine release from VTA projections in the accumbal shell. This would align with an opponent process theory of addiction (Solomon & Corbit, 1974; Koob & Le Moal, 2008; Gardner, 2011), and would help explain why excitatory output from the ventral hippocampus can have an excitatory effect on accumbal dopamine release in control conditions (Barr *et al.*, 2014; Chapter 3, Fig. 3-3A), which is *enhanced* in acute psychostimulant withdrawal (Barr *et al.*, 2014), but then has a *reducing* effect on accumbal dopamine output in protracted withdrawal (chapter 3, Fig. 3-3A). This would also implicate dysregulations within the hippocampal-mesolimbic connections as directly contributing to the dysphoric states that can prompt relapse during protracted amphetamine withdrawal (Sinha, 2007; Kitanaka *et al.*, 2008; Paliwal *et al.*, 2008; Wheeler *et al.*, 2008; Wheeler & Carelli, 2009; Koob & Volkow, 2010; Gardner, 2011; Wheeler *et al.*, 2011; Twining *et al.*, 2014; Haake *et al.*, 2018).

### **3. Final Conclusions**

There are many gaps in this overall theory that require future testing (some of which are highlighted in Table A-2). Filling in these gaps can clarify what neurobiological alterations mediate stress hypersensitivity in withdrawal, how the ventral hippocampal glucocorticoid system changes throughout the withdrawal prognosis, and what the functional and behavioral effects of these changes are.

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## Table Legend

**Table A-2: Known and unknown neurobiological alterations in the ventral hippocampus in acute (< 24 h) and protracted (2 weeks) psychostimulant withdrawal** suggest these may represent two discrete time points in the withdrawal prognosis with some neurobiological alterations that fundamentally oppose one another, in accordance with Solomon and Corbit's original opponent-process theory of motivation (1974).



**Table A-2**

<b>Effect</b>	<b>Control Conditions</b>	<b>Acute (&lt; 24) Psychostimulant Withdrawal</b>	<b>Protracted (2 weeks) Psychostimulant Withdrawal</b>	<b>Protracted (4 weeks) Psychostimulant Withdrawal</b>
<b>Basal serotonin in the ventral hippocampus</b>	---	Reduced (Barr <i>et al.</i> , 2010)	???	Restored (reduced relative to the reduction seen at 24h, but similar amounts compared to those in saline pre-treated rats) (Barr <i>et al.</i> , 2010)
<b>Stress/GR-induced serotonin release (in the ventral hippocampus)</b>	Stress/GRs induce serotonin release (Barr & Forster, 2011; Li <i>et al.</i> , 2014)	Abolished GR-induced serotonin release (Barr & Forster, 2011)	Abolished stress-induced serotonin release (Li <i>et al.</i> , 2014)	???
<b>OCT-3/SERT</b>	SERT and OCT-3 contribute to serotonin clearance and corticosterone can inhibit their function	Increased OCT-3 (expression and function) but not SERT (Barr <i>et al.</i> , 2013; Solanki <i>et al.</i> , 2016)	???	OCT-3 expression restored (Solanki <i>et al.</i> , 2016)
			Hypothesized to be restored (See chapter 4)	
<b>Serotonin release</b>	The amount of serotonin released into the vHipp was assessed by using KCl to induce local depolarization, then measuring extracellular 5-HT (Barr <i>et al.</i> , 2013)	Blunted extracellular serotonin in the vHipp in response to local KCl depolarization (Barr <i>et al.</i> , 2013)	???	???

Effect	Control Conditions	Acute (< 24) Psychostimulant Withdrawal	Protracted (2 wks) Psychostimulant Withdrawal	Protracted (4 wks) Psychostimulant Withdrawal
CRF receptor expression in the dorsal raphe nucleus (dRN)	Stress can induce serotonin release from the dRN by activating CRF receptors	Increased CRF2 receptor expression; no change in CRF1 expression (Pringle <i>et al.</i> , 2008)	CRF2 receptor expression increased at 6 weeks of withdrawal; likely to be increased at 2 and 4 weeks. CRF1 receptor expression not altered at 20h or 6wks of withdrawal (Pringle <i>et al.</i> , 2008)	
Serotonin output from dorsal raphe nucleus (dRN) to central amygdala (CeA)	In control conditions, CRF in the dRN does not alter serotonin release in the central amygdala	CRF in the dRN increases extracellular 5-HT levels at 20h WD (Scholl <i>et al.</i> , 2010)	???	???
Basal serotonin (5-HT) and serotonin metabolite (5-HIAA) in dorsal and medial raphe nucleus (d/mRN)	The medial and dorsal raphe nucleus supply the ventral hippocampus with serotonin (5-HT); 5-HIAA is a serotonin metabolite	???	No change in basal tissue [5-HT] or [5-HIAA] in mRN. In dRN, [5-HIAA] is not changed; tissue [5-HT] is increased in non-sensitized rats (but not in sensitized rats) (Scholl <i>et al.</i> , 2009)	???
Serotonin output from the raphe nucleus to the ventral hippocampus	The medial and dorsal raphe nuclei supply the ventral hippocampus with serotonin	???	???	???
Glucocorticoid receptor (GR) expression in the ventral hippocampus (vHipp)	Glucocorticoid receptors in the ventral hippocampus are thought to mediate stress responses, induce serotonin release (Barr et al., 2011), interact with interneuron inhibition/disinhibition (Figs. 1-4, 4-1), Ratio of [GR]/[MR] Attributed with Stress Sensitivity and Affect States (Chapters 2-4)	Reduced (Barr & Forster, 2011)	???	???
			Hypothesized to be restored	Hypothesized to be restored
		Unknown whether expression of cytosolic/membrane-bound or genomic/non-genomic receptors is altered		

Effect	Control Conditions	Acute (< 24) Psychostimulant Withdrawal	Protracted (2 weeks) Psychostimulant Withdrawal	Protracted (4 weeks) Psychostimulant Withdrawal
Basal corticosterone levels in the ventral hippocampus	Diurnal rhythm	Unchanged (Barr & Forster, 2011)	Unchanged (Bray <i>et al.</i> , 2016)	???
Basal plasma corticosterone levels	---	Unchanged (Barr <i>et al.</i> , 2010)	Unchanged (Bray <i>et al.</i> , 2016)	Unchanged (Barr <i>et al.</i> , 2010)
Corticosterone stress response in the ventral hippocampus	Stress increases vHipp Cort (Droste <i>et al.</i> , 2008; Bray <i>et al.</i> , 2016)	???	Enhanced (Bray <i>et al.</i> , 2016)	???
Plasma corticosterone stress response	Stress increases plasma corticosterone acutely, which is mediated by feedback inhibition (Bray <i>et al.</i> , 2016)	???	Unchanged (Bray <i>et al.</i> , 2016)	???

Effect	Control Conditions	Acute (< 24) Psychostimulant Withdrawal	Protracted (2 wks) Psychostimulant Withdrawal	Protracted (4 wks) Psychostimulant Withdrawal
<b>Inhibitory tone/ tonic inhibition in the ventral hippocampus</b>	Hypothesized to exist in control conditions (Chapters 3 & 4)	Hypothesized to be reduced (Chapter 4)	Hypothesized to be enhanced, necessitating the need for GR- mediated disinhibition (Chapter 4)	???
<b>Excitatory drive from the ventral hippocampus to the nucleus accumbens shell</b>	vHipp excitation enhances accumbal shell DA release (Barr <i>et al.</i> , 2014)	Enhanced (Barr <i>et al.</i> , 2014)	???	???
			Hypothesized to be reduced, possibly resulting from enhanced vHipp inhibitory tone, as proposed above (Chapters 3-4)	Hypothesized to be restored
<b>Excitation/inhibition balance/tone in the nucleus accumbens shell</b>	Favors inhibition (Scudder <i>et al.</i> , 2018)	Favors excitation (Scudder <i>et al.</i> , 2018)	???	???
			Hypothesized to favor <i>enhanced</i> inhibition (Chapters 3-4)	Hypothesized to be restored

Effect	Control Conditions	Acute (< 24) Psychostimulant Withdrawal	Protracted (2 wks) Psychostimulant Withdrawal	Protracted (4 wks) Psychostimulant Withdrawal
Neurogenesis	Progenitor cell proliferation	Unchanged (Barr <i>et al.</i> , 2010)	??	???
	New neuron survival	No change in survival of new (BrdU-labeled) neurons (Barr <i>et al.</i> , 2010)	??	Reduced number of BrdU+ neurons labeled directly after 2 weeks of amphetamine exposure; suggests reduced survival of new neurons (Barr <i>et al.</i> , 2010)
	Differentiation	Unchanged (Barr <i>et al.</i> , 2010)	??	Unchanged (Barr <i>et al.</i> , 2010)
	Programmed cell death	Unchanged (Barr <i>et al.</i> , 2010)	??	Unchanged (Barr <i>et al.</i> , 2010)
Behavior	Anxiety-like behavior (as measured using an elevated plus maze)	Increased (Vuong <i>et al.</i> , 2010)	Increased (Vuong <i>et al.</i> , 2010; Reinbold <i>et al.</i> , 2014; Tu <i>et al.</i> , 2014)	Increased (Barr <i>et al.</i> , 2010)
	Stress-induced behavioral arousal	???	Increased (Li <i>et al.</i> , 2014)	???

**Abbreviations:** 5-HT: serotonin; Cort: corticosterone, DA: dopamine, dRN: dorsal raphe nucleus (supplies vHipp with serotonin); GR: glucocorticoid receptor; [GR]: glucocorticoid receptor expression; MR: mineralocorticoid receptor; [MR]: mineralocorticoid receptor expression; mRN: medial raphe nucleus (supplies vHipp with serotonin); NAcS: nucleus accumbens shell; OCT-3: organic cation transporter 3 (mediates serotonin clearance); RN: raphe nucleus; SERT: serotonin reuptake transporter (mediates serotonin clearance); vHipp: ventral hippocampus. **See reference list above for full citation references.**

### Appendix 3: Support for cytosolic receptor induction of rapid non-genomic effects

In chapters 3 and 4, we propose that corticosterone's *cytosolic* glucocorticoid receptors may possess the capacity to induce rapid *non-genomic* effects (Figs. 1-4 and 4-1). We also propose that corticosterone's rapid effects (Fig. 3-3) may require activation of cytosolic receptors. This proposal contradicts the conventional assumption that corticosterone's cytosolic receptors are genomic and non-genomic receptors are membrane-bound. However, this proposal is supported by a variety of indirect and direct findings.

For example, corticosterone activation of cytosolic glucocorticoid receptors has been directly demonstrated to rapidly and reversibly inhibit ATP-induced currents within seconds in rat dorsal root ganglion cultures (Liu *et al.*, 2008). This effect was blocked by mifepristone, which has been proposed to selectively block cytosolic – but not membrane – glucocorticoid receptors (Venero & Borrell, 1999; Li *et al.*, 2014). The effect was also blocked by the phosphokinase A inhibitor H89, and was unable to be reproduced by membrane-impermeable corticosterone conjugated to BSA. Together, these findings directly attribute rapid non-genomic effects to cytosolic glucocorticoid receptors.

Ligand-binding to cytosolic glucocorticoid- and mineralocorticoid receptor *complexes* have also been found to induce non-genomic effects through dissociation of trans-activator proteins from the cytosolic complex. (Tumlin *et al.*, 1997; Croxtall *et al.*, 2002; Horvath & Wanner, 2006). The trans-activator proteins can then interact with other signaling pathways to rapidly alter signaling cascades *in vitro* and *ex vivo*. *In vitro*, activation of the cytosolic glucocorticoid

receptor complex can induce Src dissociation, which inhibits cytosolic phospholipase A2 (cPLA2) activation and arachidonic acid production (Croxtall *et al.*, 2002). Activation of the cytosolic *mineralocorticoid* receptor complex can also induce dissociation of heat shock protein 90 (Hsp90), which subsequently increases calcineurin phosphatase activity *ex vivo* (Tumlin *et al.*, 1997). Although these findings were not reproduced *in vivo* (Bisgaard *et al.*, 2001), they provide additional direct evidence that attribute non-genomic capabilities to cytosolic receptors.

Furthermore, cytosolic- and membrane-bound glucocorticoid receptors are known to have high sequence homology and may originate from the same gene transcript (Vernocchi *et al.*, 2013). Functional and structural differences between the two receptors are thought to result from alternative splicing or other posttranslational modifications (Stahn *et al.*, 2007; Stahn & Buttgerit, 2008; Oakley & Cidlowski, 2013; Rainville *et al.*, 2015; Scheschowitsch *et al.*, 2017). Indeed, the membrane *glucocorticoid* receptor has been speculated to be an isoform of the *cytosolic* glucocorticoid receptor (Scheschowitsch *et al.*, 2017). This may be true for mineralocorticoid receptors as well. For example, Karst *et al.* (2005) found that the rapid and reversible excitatory effects exhibited by activation of a mineralocorticoid receptor in the CA1 membrane could not be reproduced in brain-specific mineralocorticoid receptor knockout mice. This suggests that rapid and reversible effects elicited by membrane mineralocorticoid activation require the gene for the non-genomic mineralocorticoid receptor. Therefore, these findings indirectly link cytosolic receptors with non-genomic capabilities.

Finally, the capacity for a genomic receptor to induce non-genomic actions has been demonstrated in another member of the nuclear receptor superfamily, the Vitamin D Receptor

(VDR). This receptor is known to have a genomic binding pocket (VDR-GP) attributed with regulating VDR's genomic effects and an alternative binding pocket (VDR-AP) attributed with VDR's rapid, non-genomic responses such as opening voltage-gated calcium and chloride channels (Haussler *et al.*, 2011). Together, these findings imply that cytosolic receptors may be able to produce both genomic and non-genomic effects. Therefore, these findings support our conclusion that the rapid ability of corticosterone in the ventral hippocampus to alter accumbal dopamine release may involve activation of glucocorticoid receptors *in the cytosol*, as has been directly demonstrated by others (Croxtall *et al.*, 2000; Croxtall *et al.*, 2002; Liu *et al.*, 2010).

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**Final Note to My Committee:**

**Thank you for your invaluable help and guidance throughout my PhD endeavors!**

“Je n’ai fait celle-ci plus longue que parce que je n’ai pas eu le loisir de la faire plus courte.”

- Blaise Pascal, “Lettres Provinciales”