Investigating the Role of Glucocorticoids in Mediating Dopamine-dependent Cue-reward Learning

by

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DEDICATION

This dissertation is dedicated to my parents, Fabiola Campos and Jose Luis Lopez, and my brothers, Ivan Lopez and Jose Roberto Santos. Gracias por su apoyo. ¡Siempre juntos y si se puede!

AND

To all the empowering mentors in my life, I hope to pay it forward.

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Abstract

The way individuals respond to their surrounding environment can be advantageous or deleterious to survival. Importantly, individuals vary in their response to discrete environmental cues and this variation may be a key determinant of psychopathology. The ability of previously neutral cues to promote aberrant behavior is a hallmark of several psychiatric disorders including, addiction, post-traumatic stress disorder, eating disorders and obsessive-compulsive disorder. Thus, it is important to uncover the neural mechanisms by which such cues are able to attain inordinate control and promote psychopathological behavior. This dissertation will address the role of glucocorticoids in the attribution of incentive value to cues, a psychological process that transforms such cues into powerful motivators of behavior, that may be adaptive/ maladaptive. Additionally, it will focus on the relationship between glucocorticoids and dopamine, the latter of which is critical to the process of incentive salience attribution.

Glucocorticoids are primarily recognized as the main hormone secreted in response to stress but are known to exert their effects across the body and the brain, and to affect learning and memory, cognition, and reward-related behaviors, among other things. Our understanding of this hormone in incentive learning stems from work demonstrating differences in peripheral levels of glucocorticoids in rats that learn a predictive cue-reward association (goal-trackers) compared to those that also attribute incentive value to the cue (sign-trackers). However, whether these differences pertained to differences in stress-responsivity was unknown. In Chapter 2, we assessed

neuroendocrine and behavioral profiles associated with negative valence in male rats that show a preference for incentive learning (sign-trackers), compared to those that purely learn the cue-reward association (goal-trackers). We found that they do not differ in negative valence indices; rather differences in neuroendocrine measures, like glucocorticoids, can be attributed to distinct cue-reward learning styles. In Chapter 3, we studied whether pharmacological alterations in glucocorticoid levels prior to training affected the goal- and sign-tracking tendencies of male and female rats. We found that, in males, elevated levels of glucocorticoids promote incentive learning, whereas in females there is an attenuation that is reversed when treatment is removed. Finally, in chapter 4, we captured peripheral and brain levels of glucocorticoids and dopamine, specifically within the nucleus accumbens shell of male and female goal- and sign-trackers. Glucocorticoids and dopamine differed based on sex and preferred cue-reward learning strategies; but no significant relationship was found between accumbens glucocorticoids and dopamine. Collectively, these studies serve as evidence for a role of glucocorticoids, beyond negative valence systems, and in particular in cue-reward learning. It appears that while glucocorticoids influence the propensity to attribute incentive value to reward cues, the state under which they are acting may impact their interaction with dopamine and subsequent influence on behavior. This work will serve as a foundation for future studies probing the role of glucocorticoids in cue-motivated behaviors relevant to psychopathology.

Chapter 1

General Introduction

Note: Portions of the text and the figure within Chapter 1 have appeared previously in print (Lopez & Flagel, 2020, Stress) and are reproduced here with permission from the publisher.

Daily, we are surrounded by an overwhelming amount of cues (e.g., smells, sounds, sights) in our environment. These cues are often associated with prior experiences, and consequently, attain the ability to influence our behavior in adaptive ways. For example, suppose we were in a state of hunger. In that case, a cue signaling the availability of food can advantageously elicit approach behavior towards the valuable resource. It is through Pavlovian conditioning (Pavlov, 1927) that both humans and animals learn to associate discrete environmental cues with biologically relevant stimuli like food, sex, or threat (Rescorla, 1988). By learning to associate a discrete cue with a positive (e.g., Jenkins & Moore, 1973) or negative stimulus (e.g.,Rescorla, 1968), the otherwise neutral cue attains predictive value and can facilitate adaptive behavior. However, predictive cues can also attain the ability to function as powerful "lures" of aberrant behavior, which can be maladaptive; and they do so through the psychological process of incentive salience attribution (Robinson & Berridge, 1993). Once transformed into incentive stimuli – via the attribution of incentive salience – cues and their sensory qualities become especially salient. For example, reward-associated cues can become

attractive, attention-grabbing, and desired as if they were themselves the reward (Berridge, 2001; Bindra, 1978; Bolles, 1972; Toates, 1986). One such occurrence is the undue influence drug-paired cues can have over behavior for individuals with drug addiction. Exposure to such cues often results in relapse (Robinson & Berridge, 1993; Tomie, 1996). Similarly, the extent to which trauma-related cues become imbued with excessive salience for individuals with post-traumatic stress disorder (PTSD) and the comorbidity of PTSD and addiction, have been discussed in the context of motivational salience attribution (Maria-Rios & Morrow, 2020).

Importantly, there is individual variation in the extent to which cues are attributed with incentive salience (Robinson & Flagel, 2009). In fact, individual differences in cue-learning were described as early as 1937 by Karl Zener. Derived from Pavlov's classic experiments (Pavlov, 1927), Zener demonstrated that Pavlovian conditioning in unharnessed dogs (i.e., freely moving) resulted in distinct conditioned responses (CRs) to the Pavlovian cue (Zener, 1937). Upon presentation of a tone-cue (conditioned stimulus, CS), some dogs would direct their behavior toward the food-pan, where the food-reward (unconditioned stimulus, US) was going to be delivered; whereas other dogs seemed to be attracted to the CS itself. The former CR was eventually termed "goal-tracking" (Boakes, 1977), in reference to approach behavior directed toward the food tray, or goal location, during the presentation of the food-predictive lever-CS. In contrast, "sign-tracking" (Brown & Jenkins, 1968; Hearst & Jenkins, 1974), referred to behavior directed towards the CS, a term first used to describe pigeons' behavior (i.e., pecking) directed toward a key-light-cue that signaled the delivery of food.

In recent years, Flagel and colleagues have implemented the use of the goal-tracker/ sign-tracker animal model, allowing researchers to parse two psychological processes, predictive- and incentive-learning, by capturing differences in the CR to a Pavlovian cue in rats (Robinson & Flagel, 2009). For both goal-trackers (GTs) and sign-trackers (STs), the CS serves as a predictor of reward, but for sign-trackers the cue also become an incentive stimulus, or a "wanted" target (Berridge et al., 2009). Previously, the predictive and incentive qualities of a CS were considered to be intertwined (e.g., de Wit & Stewart, 1981). Thus, while the neurobiology of cue-reward learning was beginning to be elucidated (for review, see Cardinal et al., 2002), the interpretation of such studies was confounded by the inability to parse predictive vs. incentive learning mechanisms. The use of the GT/ST model has since been exploited to identify the neurobiological substrates of goal-tracking (i.e., predictive) vs. sign-tracking (i.e., incentive) behaviors. Indeed, these distinct learning strategies are also neurobiologically dissociable (e.g., Campus et al., 2019; Flagel, Clark, et al., 2011; Pitchers, Kane, et al., 2017). Specifically, goal-tracking is thought to rely on cortical "top-down" circuits (Campus et al., 2019; Sarter & Phillips, 2018), while sign-tracking is presumed to be driven by subcortical "bottom-up" mechanisms (Flagel, Cameron, et al., 2011; Haight et al., 2017; Yager et al., 2015). Additionally, we know that the sign-tracking response, and not goal-tracking, is dependent on dopamine (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012).

The majority of prior studies have focused on the role of the classic "motive circuit" (Kalivas & Volkow, 2005; Kelley et al., 2005) and positive valence systems (RDoC) in goal- and sign-tracking behavior (Flagel & Robinson, 2017; Kuhn, 2018).

However, an early study identified a potential role for glucocorticoids in mediating individual differences in cue-reward learning (Flagel et al., 2009); and some studies have demonstrated that goal- and sign-trackers respond differently to an aversive CS (Morrow et al., 2011; Morrow et al., 2015). Thus, there is some evidence to suggest that GTs and STs may be distinctively sensitive to negative valence events.

The way individuals respond to environmental stimuli, positive and/or negative, may be indicative of vulnerability to psychopathology. Accordingly, the GT/ST model is a valuable tool to investigate both the psychological and neurobiological processes involved in the individual variation in cue-motivated behaviors, including those resembling pathological behaviors. Following is a review of the behavioral traits and neurobiology associated with predictive vs. incentive cue-learning or the goal-tracker/sign-tracker animal model to date. In addition, there is a brief overview of the role of glucocorticoids in the stress response, learning and memory, and reward learning; all in support of a framework postulating a role for this hormone in mediating dopamine-dependent cue-reward learning (i.e., sign-tracking).

The goal-tracker/ sign-tracker animal model

The GT/ ST model consists of exposing rats to a Pavlovian conditioned approach (PavCA) paradigm. An illuminated lever-CS is presented for 8 secs and precedes the impending delivery of a food reward-US into a food-cup. With training, and similar to prior observations (e.g., by Zener, Boakes, Hearts and Jenkins), different conditioned responses (CRs) emerge, goal-tracking and sign-tracking (Robinson & Flagel, 2009). During the CS presentation, GTs direct themselves to the food-cup, in anticipation of the US; whereas STs interact with the CS (e.g., chewing, gnawing). When the lever-CS is

retracted, both phenotypes retrieve and consume the food reward. Some rats, intermediate responders (IRs), vacillate between the two CRs with no preference for either learning strategy. As an incentive stimulus, the food-cue attains unique properties (Robinson & Berridge, 1993), enabling it to bias attention and elicit approach behavior (e.g., Harmer & Phillips, 1998), act as a conditioned reinforcer (e.g., Di Ciano et al., 2007; Taylor & Horger, 1999), and energize ongoing actions (e.g., Wyvell & Berridge, 2001). This transformation of the reward cue into a “motivational magnet” occurs to a greater degree in STs (Berridge et al., 2009). The first of these properties is measured by CS approach behavior, and thus, is captured by PavCA and the emerging conditioned response of sign-trackers (Flagel et al., 2008). The second property can be measured by a conditioned reinforcement test, wherein a nose poke into an "active" port results in the presentation of the lever-CS, and a nose poke into an "inactive" port has no consequence. Relative to GTs and IRs, STs poke into the "active" port more readily and interact with the lever to a greater extent (Robinson & Flagel, 2009). That is, the cue serves as a more effective conditioned reinforcer for STs. Finally, the third property is measured by Pavlovian instrumental transfer (PIT), where the Pavlovian reward-associated cue enhances an operant response for the delivery of the same reward (i.e., specific PIT) or a different reward (i.e., general PIT). For example, first, a Pavlovian association is made between a tone-CS and a food reward. Second, rats learn to press a lever to deliver the same food reward (i.e., operant conditioning), and third, when the CS is presented, this enhances lever pressing for the food reward. (e.g., Estes, 1948). Given a lever-CS is used for the characterization of goal- and sign-tracking, and this confounds with the methodology of PIT, the third property is not met in the "traditional"

way. Using this model, however, it has been demonstrated that indeed reward-cues invigorate a motivational state to a greater extent in STs, relative to GTs. Specifically, STs are enticed by food- and drug-associated cues and, as a result, engage in greater food- or drug-seeking behavior, which is an operant response (Saunders et al., 2013; Yager & Robinson, 2010). In summary, for sign-tracker rats and not goal-trackers, Pavlovian cues attain incentive motivational value; and this is confirmed by the acquisition of three fundamental properties of an incentive stimulus (Flagel et al., 2009).

The co-occurrence of behavioral traits with the propensity to goal- or sign-track in response to reward cues

Interestingly, the propensity to either goal-track or sign-track to a food-cue is accompanied by several "vulnerability" traits relevant to psychopathology. That is, in characterizing sign-trackers, additional traits unfold, including, an enhanced propensity to attribute incentive salience to drug-paired cues (Flagel et al., 2010; Uslaner et al., 2006; Yager et al., 2015; Yager & Robinson, 2013), enhanced psychomotor sensitization to cocaine (Flagel et al., 2008), more motivation to take drugs following limited drug experience (Saunders & Robinson, 2011), a higher propensity for reinstatement of drug-seeking behavior, or relapse (Saunders & Robinson, 2010, 2011; Saunders et al., 2013) (but also see Kawa et al., 2016), greater impulsive action (Lovic et al., 2011), an enhanced and exaggerated fear response to discrete aversive stimuli (Morrow et al., 2011; Morrow et al., 2015), and poor attentional control (Paolone et al., 2013). In contrast, goal-trackers show a greater sensitivity to contextual cues. For example, relative to STs, GTs show greater drug-seeking behavior in response to contextual cues (Saunders et al., 2014), and an enhanced fear response to an aversive

context (Morrow et al., 2011). Additionally, GTs show greater relapse of drug-seeking behavior triggered by discriminative stimuli, or occasion setters (Pitchers, Phillips, et al., 2017). Thus, the GT/ST model is believed to capture more than differences in cue-learning strategies; rather, it captures behavioral “endophenotypes” that may constitute two different pathways of vulnerability to psychopathology.

The neurobiological substrates implicated in goal-tracking vs. sign-tracking

Besides GTs and STs being behaviorally distinguishable, several studies have demonstrated that different neural circuits are engaged in response to reward-cues in GTs vs. STs. As mentioned above, it is believed that GTs have greater "top-down" cortical control, and a deficit thereof is thought to contribute to the sign-tracking response (Campus et al., 2019; Sarter & Phillips, 2018). In contrast, sign-tracking is presumed to be driven by subcortical "bottom-up" circuits (Flagel, Cameron, et al., 2011; Haight et al., 2017; Yager et al., 2015), and we know it is dopamine-dependent (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012). Thus, these extreme behavioral phenotypes, or cue-learning strategies, are hypothesized to arise from an imbalance of "top-down" control vs. "bottom-up" drive (Campus et al., 2019; Flagel, Cameron, et al., 2011; Haight et al., 2017).

“Top-down” control

The prefrontal cortex (PFC) is critical in mediating "top-down" executive control (Weissman et al., 2006), and its ability to achieve focus and eliminate "distractions" makes it an important mechanism involved in goal-directed behaviors (e.g., Mihindou et al., 2013). Not surprisingly, relative to STs, GTs perform better in tasks that rely heavily on this type of control; for example, tasks that demand impulse control (Flagel et al.,

2010; Lovic et al., 2011) or sustained attention (Paolone et al., 2013). In this regard, cholinergic modulation within the PFC is postulated as an important component driving these opposing cognitive styles between GTs and STs (Sarter & Phillips, 2018). In support, STs exhibit attenuated levels of acetylcholine (Ach) during a sustained attention task, and their performance on the task can be improved with the administration of an ACh receptor agonist (Paolone et al., 2013). Further, and relevant to Pavlovian conditioning, GTs show an increase of PFC ACh levels with the presentation of the CS, while no change is observed in STs; instead, STs show an increase in PFC DA (Pitchers, Kane, et al., 2017). Thus, these findings emphasize the relationship between increased cortical ACh levels and goal-tracking, and the lack thereof in STs. Additionally, the contrasting increase of DA for STs highlights how distinct modulators, in this case, within the same brain region, are contributing to goal- vs. sign-tracking behaviors.

Another mechanism that may be contributing to the differential engagement of the PFC in goal- vs. sign-trackers is serotonin. Increased levels of serotonin in rat PFC tissue (Tomie et al., 2004), as well as, greater binding levels to its receptors (Tomie et al., 2003) have been observed following appetitive Pavlovian conditioning. Notably, depletion of serotonin within the forebrain, including the PFC, results in elevated cue-directed behavior (i.e., sign-tracking) (Winstanley et al., 2004). Yet again, supporting the idea that sign-tracking involves a deficit in cortical engagement, that may involve serotonin levels.

The PFC has been implicated in goal- vs. sign-tracking in a circuit-specific way. The presentation of a food- (Flagel, Cameron, et al., 2011; Haight et al., 2017) or drug-

cue (Yager et al., 2015) induces distinguishable c-fos expression, a marker of neural activity, across the brains of STs relative to GTs. Follow up studies have focused on determining the specific influence of brain regions and circuits identified via these c-fos comparisons. For example, a “suppressing” role has been identified for the paraventricular nucleus of the thalamus (PVT), with pharmacological lesions resulting in increased expression of CS-directed behaviors for GTs (Haight et al., 2015). Interestingly, in GTs, PFC activity strongly correlates with that of the PVT (Flagel, Cameron, et al., 2011); and it has been demonstrated that chemogenetic inhibition (i.e., “turning-off) of the PrL-PVT circuit results in increased attribution of incentive value to a reward-cue for GTs, whereas, for STs, stimulation (i.e., “turning-on”) of this pathway decreases the incentive value (Campus et al., 2019). In summary, cortical control appears to sufficiently impact the propensity to sign- and goal-track to a Pavlovian cue; specifically, it appears turning-off the PrL-PVT circuit impels rat behavior toward CS-directed behaviors (i.e., incentive learning), while turning-on the circuit dissuades them from doing so. It is believed, however, that there is a subcortical "bottom-up" drive that is concurrently dictating such cue-motivated behaviors.

“Bottom-up” drive

Unlike GTs, with the presentation of the CS, in STs, there is greater c-fos expression in subcortical brain regions, including the striatum, amygdala, thalamus, and hypothalamus (Flagel, Cameron, et al., 2011; Haight et al., 2017). Further, for STs, there are strong correlations of activity between subcortical regions like the lateral hypothalamus (LH), nucleus accumbens (NAc), and PVT. Interestingly, disrupting the suppression effects of top-down control over the attribution of incentive value is

accompanied by an increase in subcortical drive. For example, “turning-off” the PrL-PVT circuit (Campus et al., 2019) in GTs is accompanied by an increase in extracellular DA within the NAc shell (NAcS) (Campus et al., 2019).Thus, the PVT appears to be critical integrator of both cortical (i.e. PrL) and subcortical (i.e., NAcS) circuits, making it a unique structure that, in turn, dictates motivated behavior via output structures like the NAc (Haight & Flagel, 2014). Subcortical inputs from the LH to the PVT are thought be especially important for mediating the sign-tracking response (Haight & Flagel, 2014; Haight et al., 2017). In support, recent studies have demonstrated that the LH is necessary for the development of a sign-tracking conditioned response (Haight et al., 2020). In this regard, orexinergic input from the LH to the PVT seems to be especially relevant (Haight & Flagel, 2014), as blocking orexin receptors within the PVT results in reduced expression of sign-tracking and an attenuation of the conditioned reinforcing properties of the CS (Haight et al., 2020). Together, these findings argue in favor of the “bottom-up” drive that is presumed to underlie incentive value attribution. Further, and in alignment with the influence of the PrL-PVT circuit on NAc extracellular DA levels, it has been postulated that the LH-PVT circuit mediates output from the PVT to the NAc (Haight et al., 2020). Thus, the LH-PVT-NAc circuit may be an overriding force opposing PrL-PVT control of incentive value attribution.

Dopamine

Dopamine (DA) within the NAc is critical for the attribution of incentive value, with sign-tracking, but not goal-tracking, being DA-dependent (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012). While it is well accepted DA release within the NAc increases in response to discrete reward-cues (e.g., Di Ciano et al., 1998), there are

two major theories in place regarding the role of DA (Berridge & Robinson, 1998; Schultz et al., 1997). Schultz and colleagues propose that DA mediates the prediction of reward by encoding, or updating, new predictions when the outcome of reward deviates from what is expected, and this is referred to as the “prediction error” theory (Schultz et al., 1997). In contrast, Berridge and Robinson postulate that DA encodes the incentive motivational value of reward-cues (Berridge & Robinson, 1998). Given the GT/ST model parses predictive vs. incentive learning, its use in addressing this debate has been integral. First, it revealed that throughout the course of Pavlovian conditioning, DA-related mechanisms within the NAc are distinctly expressed between GTs and STs (Flagel et al., 2007). Per the “prediction error” theory, there is an initial rise in phasic DA release in the NAc in response to the reward (US), and upon the learned CS-US association, there is a shift in DA response to the predictive CS (Schultz et al., 1997). Flagel and colleagues found that this shift in DA from the US to the CS only occurs in STs, not GTs (Flagel, Clark, et al., 2011). Given that predictive value is attributed to the cue by both GTs and STs, DA is thereby believed to explicitly encode the incentive value (Flagel, Clark, et al., 2011). Further, pharmacological studies using a nonselective DA receptor antagonist, Flupenthixol, showed that DA is necessary for sign-tracking, but not goal-tracking behavior and this is apparent with both systemic (Flagel, Clark, et al., 2011) and intra NAc administration (Saunders & Robinson, 2012). Together, this work has established that DA is necessary for the sign-tracking response and specifically that DA within the NAc is crucial for incentive value attribution.

In conclusion, the GT/ST animal model captures neurobehavioral endophenotypes of relevance to cue-driven psychopathologies. Determining the

neurobiological substrates driving individual differences in response to environmental stimuli will increase our understanding of the pathological behaviors manifested, for example, in addiction and PTSD; and this preclinical animal model can be exploited to do so. While it is hypothesized that relying more on either “top-down” or “bottom-up” circuits contributes to goal-tracking vs. sign-tracking, dopamine is pivotal for the sign-tracking conditioned response, and in fact, plays a significant role for some of its co-existing traits like the propensity to “relapse”(Saunders et al., 2013). Dopamine and its relevance to positive valence systems, like the response to rewards (Berridge, 2001), has long been studied. However, it has also been reported that dopamine within the NAc increases in response to stressors (e.g.,Kalivas & Duffy, 1995) and with the administration of glucocorticoids (e.g., Piazza et al., 1996). It has been postulated that dopamine and glucocorticoids, although often associated with negative valence, interact to potentiate drug-motivated behaviors (for review, see Piazza & Le Moal, 1996). However, much remains to be determined regarding the role of glucocorticoids in dopamine-dependent cue-motivated behaviors.

Glucocorticoids

Glucocorticoids (GCs), the end product of the hypothalamic-pituitary-adrenal (HPA) axis, circulate the body and brain of both humans and animals and rise in response to environmental challenges (for review, see Herman et al., 2016). GCs are classically recognized as the “stress” hormone (J. P. Henry, 1992), but play a wide-ranging role in adaptive physiology and behavior (Myers et al., 2014). For example, GC function has been implicated in metabolism (Rose et al., 2010; Vegiopoulos & Herzig, 2007), inflammation (De Bosscher & Haegeman, 2009; Scheschowitsch et al., 2017),

immune response (Cain & Cidlowski, 2017), development (Busada & Cidlowski, 2017; De Kloet et al., 1988)**,** memory (Roozendaal, 2000; Sandi & Pinelo-Nava, 2007), cognition (de Kloet et al., 1999; Sandi, 2013), and reward-processing (Leal & Moreira, 1997; Piazza et al., 1989). Therefore, it is perhaps not surprising that GC function has been recognized in the pathophysiology and neurobiology of disease and affective disorders for more than 70 years (see McEwen & Akil, 2020; Selye, 1955). Hyper- and hypo-concentrations of GCs, as well as altered GC receptor expression levels, have been reported in individuals with depression, schizophrenia, and PTSD (Akil, 2005; Muck-Seler et al., 1999; Szeszko et al., 2018). Further, individuals with excessive glucocorticoid secretion (e.g., Cushing's syndrome) (Sonino et al., 2010), or those prescribed GCs (e.g., for treating inflammation), often report depressed mood (Marques et al., 2009). While there is an abundance of data in support of a relationship between glucocorticoid function, emotional state, and maladaptive behavior, the point of intersection remains to be determined.

Glucocorticoid function and the stress response

Historically, HPA axis has been viewed as the primary biological system activated by stress (e.g., Dallman & Jones, 1973). Upon perceiving a stressor (see Figure 1.1), neural signals are elicited throughout the brain, including the prefrontal cortex, hippocampus, amygdala and brainstem (for review, see Herman & Cullinan, 1997, and Figure 1.1). This information converges at the paraventricular nucleus (PVN) of the hypothalamus via direct projections or indirectly through the bed nucleus of the stria terminalis (BNST) and neighboring hypothalamic nuclei (for review, see Herman et al., 2003). The HPA axis is thereby activated by secretion of corticotropin-releasing

hormone (CRH) and arginine vasopressin (AVP) from the PVN. Adrenocorticotropic hormone (ACTH) is subsequently released from the pituitary, and ultimately, GCs are synthesized and released from the adrenal glands (for review, see Herman et al., 2016; Spencer & Deak, 2017). Even in the absence of stress, GCs - cortisol in humans and corticosterone in rodents - circulate both peripherally and centrally in fluctuating concentrations that follow an ultra-radian and circadian rhythm (Kalsbeek et al., 2012; Lightman & Conway-Campbell, 2010; Qian et al., 2012; Sarabdjitsingh et al., 2010; Spiga et al., 2014). Approximately 85% of circulating GCs are inactive and bound to a glycoprotein, corticosteroid-binding globulin (CBG) (for review, see Moisan et al., 2014). At baseline, ~ 5% of GCs are free to enter the brain, however, under circadian peak or stress levels this percentage is increased (McEwen et al., 1968; Qian et al., 2012). GCs act upon two types of ligand-gated receptors, Type I-mineralcorticoid receptors (MRs) and Type II-glucocorticoid receptors (GRs) (for review, see Evans & Arriza, 1989). MRs are considered the low capacity, high-affinity receptor and are activated and occupied by baseline levels of GCs; whereas GRs are considered the high capacity, low-affinity receptor and are activated by circadian peak and stress levels of GCs (Herman et al., 1989; Reul & de Kloet, 1985; Reul et al., 1987). Unlike MRs, GRs are expressed in most cells throughout the body (for review, see de Kloet et al., 2000). GRs are also ubiquitously expressed throughout the brain with high levels of expression in the lateral septum, hippocampus, nucleus tractus solitarius, amygdala, hypothalamus, locus coeruleus, cerebellum, prefrontal cortex (PFC), ventral tegmental area (VTA), paraventricular nucleus of the thalamus (PVT), and striatum (Ahima & Harlan, 1990; Chao et al., 1989; Fuxe, Harfstrand, et al., 1985; Fuxe, Wikstrom, et al., 1985; Jaferi &

Bhatnagar, 2006; Reul & de Kloet, 1985 also see Figure 1.1). In contrast, MRs are mainly concentrated in the hippocampus, amygdala, PFC and PVT (Chao et al., 1989; Jaferi & Bhatnagar, 2006; Reul & de Kloet, 1985). GCs are lipophilic and bind primarily to MR/GRs within the cytoplasm of cells, including neurons, and are subsequently shuttled into the nucleus (Madan & DeFranco, 1993; Scheschowitsch et al., 2017). GCs can also bind to membrane-associated glucocorticoid receptors that have distinct properties from those that are cytoplasmic (Strehl & Buttgereit, 2014). The effects of GR and MR activation can be fast and non-genomic, or slow, genomic, and long lasting (Groeneweg et al., 2011; Scheschowitsch et al., 2017). One of the major roles of GC-GR interaction, however, is to protect us from further stress. The stress response is regulated via a negative feedback loop, which includes GC-GR interaction within the anterior pituitary, hypothalamus, hippocampus, and PFC (Akil, 2005; De Kloet & Reul, 1987; Herman & Mueller, 2006; Reul & de Kloet, 1985). With the initial rise of GCs in response to stress, MRs become occupied. At peak levels, MRs are saturated and GRs become bound. GR activation prevents GCs from rising to levels that are threatening to homeostasis by inhibiting further synthesis and secretion of CRH and ACTH (for review, see Gjerstad et al., 2018). The involvement of each biological mediator of the HPA axis varies depending on the nature (e.g. duration and intensity) of the stressor and the state of the organism (e.g., previous stress history) (for review, see Joels & Baram, 2009). Ultimately, GC function, among other things, is central to our ability to cope with stress, and dysregulation of the HPA axis at any level can be deleterious to an organism.

Glucocorticoids in learning and memory

Acquiring salient information (learning) and recalling it (memory) are fundamental processes underlying an individual's response to the surrounding environment. The relationship between glucocorticoid signaling and learning and memory has been a primary focus of stress neurobiology research for decades (Lupien & McEwen, 1997). This work has demonstrated that glucocorticoids can act to either improve or impair learning and memory, with the effects dependent, at least in part, on the level of circulating glucocorticoids (McEwen & Sapolsky, 1995). For example, corticosterone alters long-term potentiation (LTP) in hippocampal neurons (Bennett et al., 1991; Dubrovsky et al., 1987), but does so in an inverted-U shaped manner (Diamond et al., 1992; Pavlides et al., 1993). That is, low concentrations of GCs increase LTP, while high concentrations decrease LTP. It is not surprising, therefore, that an increase in the induction of LTP is mediated by MR activation, whereas a decrease is mediated by GR activation (Conrad et al., 1999; Pavlides et al., 1995). As expanded upon below, dose, timing, and receptor specificity are all critical factors mediating glucocorticoid effects on learning and memory, and the impact of each is dependent on the behavior being assessed.

Spatial learning and memory

Much of the work examining the effects of glucocorticoids on learning and memory has centered around spatial tasks such as the Morris Water Maze, which is known to rely on the hippocampus (e.g., Morris et al., 1990). For example, administration of corticosterone immediately following each training session of acquisition improves long-term retrieval on the Morris Water Maze task, but in an experience-dependent manner (Sandi et al., 1997). That is, experimentally-induced

endogenous corticosterone levels were identified as a critical factor determining the cognitive consequences (Sandi et al., 1997). On the other hand, chronic exposure (3 months) to corticosterone (via pellet implants) prior to training impairs learning on a Morris Water Maze task, and similar impairments were evident following pre-exposure to chronic social stress (Bodnoff et al., 1995). Given the density of glucocorticoid receptors in the hippocampus, it is not surprising that spatial learning is also affected by blocking central MRs and GRs via intracerebroventricular administration of selective antagonists (Oitzl & de Kloet, 1992; Oitzl et al., 1998). The behavioral effects reported in these studies suggest a differential role for MRs vs. GRs in spatial learning and memory, with MRs mediating spatial learning strategies and GRs playing a critical role in the consolidation of spatial information (Oitzl & de Kloet, 1992; Oitzl et al., 1998). Importantly, as with corticosterone administration, GR blockade has been shown to both improve and impair cognitive function, with effects largely dependent on the treatment regimen and timing of administration (e.g., Oitzl et al., 1998). Together, these studies highlight an important role for GC function in spatial learning and memory, and reveal the complexities of studying such a ubiquitous and wide-ranging physiological system.

Stimulus-response learning

In addition to spatial learning, GCs have also been implicated in stimulus-response learning (e.g., Atsak et al., 2016; Schwabe et al., 2010). On the circular hole board (CHB) task rodents are trained to locate an open hole or exit using either distant cues, which are reflective of spatial learning, or proximal cues, which signify stimulus-response learning. When rodents are stressed or administered corticosterone prior to a test of the "preferred" learning strategy (i.e. spatial vs stimulus-response), they rely

mainly on stimulus-response learning (Schwabe et al., 2010). Further, blocking MRs prevents stimulus-response learning and rescues spatial learning strategies, but not performance (Schwabe et al., 2010). Importantly, unlike hippocampal-dependent spatial learning, stimulus-response learning relies on the dorsal striatum (Packard & McGaugh, 1996; Packard & Wingard, 2004). Thus, these findings highlight a role for GCs in learning and memory in brain regions outside of the hippocampus.

Consistent with the rodent work described above, humans also exhibit a strategy-shift from spatial to stimulus-response learning following stress (Schwabe et al., 2008) or the administration of glucocorticoids (Guenzel et al., 2014; Schwabe et al., 2009). Specifically, individuals with a self-reported history of "high" chronic stress are more likely to exhibit a stimulus-response learning strategy relative to those with a history of "low" chronic stress (Schwabe et al., 2008). The same patterns follow exposure to an acute psychosocial stressor, and individuals who exhibit a stress-induced stimulus-response strategy tend to show greater cortisol levels during the spatial learning task (Schwabe et al., 2007). Further, administration of hydrocorticosterone prior to a stimulus-response task has been shown to enhance performance (Guenzel et al., 2014). Together, both animal and human data, suggest that GCs influence learning strategies and do so in a brain region-specific manner (e.g., Iaria et al., 2003).

Pavlovian conditioning

Glucocorticoids also play a role in Pavlovian associative learning. Most research in this regard has centered around fear conditioning (for review, see Kim & Jung, 2006), which consists of a single or multiple sessions in which a tone (conditioned stimulus,

CS) predicts the delivery of a footshock (unconditioned stimulus, US). Following associative learning, the tone-CS comes to elicit the conditioned response (CR) of freezing behavior (Fanselow, 1980). This conditioning experience elicits a rise in corticosterone (Marchand et al., 2007). Under these experimental conditions, a rise in corticosterone is expected, as acute stressors including, footshock, restraint stress, and forced swim test are known to increase corticosterone in rodents (e.g., Hueston et al., 2011). Importantly, however, the rise in corticosterone is greater in rodents that received paired presentation of the cue (CS) and footshock (US), relative to those that received random presentation of the CS and US (e.g., Marchand et al., 2007). Consistent with these findings, exposure to stress will facilitate the CS-US association (Shors et al., 1992). For example, exposure to acute stress (i.e. tail shock) *prior to* a conditioning session results in an increase in the magnitude of a CS (white noise)-elicited eye-blinking CR, and this effect appears to be corticosterone-dependent (Beylin & Shors, 2003; Shors, 2001). That is, the effect of stress is no longer apparent following adrenalectomy (Beylin & Shors, 2003). Interestingly, administration of corticosterone *after* a conditioning session will enhance the CS-elicited CR observed on subsequent sessions (Lesuis et al., 2018; Zorawski & Killcross, 2002). GC function, therefore, seems to be involved in both establishing a relationship between discrete environmental cues (CS) and aversive stimuli (US), and in expressing the corresponding behavioral response (CR).

Although much less research has investigated a potential role for GCs in appetitive Pavlovian conditioning, there is some supporting evidence (Tomie et al., 2002; Tomie et al., 2004). Following a Pavlovian conditioning session consisting of a

lever-CS paired with food-US, Tomie and colleagues (Tomie et al., 2002; Tomie et al., 2004) demonstrated an increase in corticosterone levels, which was apparent following either the 1st or 20th conditioning session. Similar to the data described above for fear conditioning, the rise in corticosterone was greater in rats that received paired presentations of the lever-CS and food-US, relative to those that received random presentations. GC function, therefore appears to play a role in both aversive and appetitive Pavlovian conditioning. The exact mechanism remains to be determined, but it is presumed that both overlapping and distinct processes are involved in the GC-mediated effects on aversive vs. appetitive conditioning. Below, one possible mechanism in the context of appetitive Pavlovian conditioning will be discussed.

Glucocorticoids in reward and dopamine

Although we lack a specific understanding of the role of GCs in appetitive Pavlovian conditioning, there is a large body of literature in support of a role for GCs in reward-related behaviors. Much like stress, exposure to rewards such as food, sex, and drugs elicit a rise in corticosterone (for review, see Piazza & Le Moal, 1997). In fact, it has been suggested that corticosterone itself is reinforcing, as rats willingly self-administer stress levels of corticosterone in a dose-dependent manner (Deroche et al., 1993; Piazza et al., 1993). Further, both stress (e.g., restraint, footshock, tailshock, isolation) (Abercrombie et al., 1989; Hall et al., 1998; Kalivas & Duffy, 1995; Puglisi-Allegra et al., 1991) and administration of corticosterone (Imperato et al., 1989; Piazza et al., 1996) have been reported to increase dopamine within the nucleus accumbens (NAc), a primary locus for reward-processing; and corticosterone dose-dependently affects the reinforcing properties of drugs of abuse (Deroche et al., 1997) as well as the

locomotor response to drugs (i.e. sensitization) (Cador et al., 1993; Marinelli et al., 1997). Together, these findings formed the foundation for the "pathophysiological chain" framework which was put forth by Piazza and LeMoal more than 20 years ago to explain individual differences in drug-taking behaviors of relevance to substance use disorder (Piazza & Le Moal, 1998; Piazza & Le Moal, 1996). At baseline, corticosterone and dopamine concentrations are typically low, as is the propensity to self-administer drugs. Under stress, however, the pathophysiological chain is triggered, and increased levels of corticosterone and dopamine are thought to interact in the NAc and, in turn, affect the reinforcing effects of drugs of abuse. Importantly, within this framework, individual differences are accounted for, as the initial rise in corticosterone or heightened sensitivity to the effects of this hormone could be inherently present in some individuals or induced by stress in others (Piazza & Le Moal, 1996). Indeed, this framework was based in large part on an animal model that captured such individual differences, as described briefly below.

Individual differences

Like humans, only some rats readily self-administer drugs of abuse, and Piazza and colleagues showed that this tendency to take drugs could be predicted by individual differences in response to a novel environment (Piazza et al., 1989). That is, high-responder (HR) rats that exhibit a greater locomotor response to a novel environment have an increased propensity to acquire drug self-administration relative to low-responder (LR) rats that exhibit low levels of novelty-induced locomotion (Piazza et al., 1990; Piazza et al., 2000). These distinct patterns of drug self-administration between HRs and LRs are apparent across drug classes, including psychostimulants (Marinelli &

White, 2000; Piazza et al., 1989), nicotine (Suto et al., 2001), morphine (Ambrosio et al., 1995) and ethanol (Nadal et al., 2002). Thus, these individual differences do not appear to be a function of drug-induced effects *directly* on the dopamine system. Rather, the behavioral characteristics of high- and low-responder rats appear to be driven largely by GC function. Relative to LRs, HRs exhibit a greater and more prolonged corticosterone response to a novel environment (Piazza et al., 1990; Piazza, Maccari, et al., 1991). This corticosterone profile has been attributed to lower GR mRNA expression in the hippocampus, or a faulty negative feedback system in HR rats (Kabbaj et al., 2000). Further, experimenter-administered corticosterone increases drug self-administration of LRs, the less vulnerable or "resistant" phenotype; but decreases drug self-administration of HRs, the more vulnerable phenotype (Piazza, Maccari, et al., 1991).These phenotype-dependent effects of corticosterone on drug-taking behavior have been attributed to inherent differences in circulating levels of corticosterone which, in turn, were postulated to interact with the dopamine system (Piazza, Maccari, et al., 1991).

Differences in DA transmission do indeed accompany the behavioral phenotypes of HRs and LRs. Relative to LRs, HRs have higher stress- and drug-induced DA within the nucleus accumbens, and, importantly, these differences are dependent on corticosterone, as they are not apparent following adrenalectomy (Rouge-Pont et al., 1998). In fact, adrenalectomy decreases nucleus accumbens DA levels in HRs such that they are indistinguishable from LRs. Beyond the nucleus accumbens, HRs and LRs also differ in stress-induced dopamine-metabolite/dopamine ratios in the dorsal striatum and prefrontal cortex (Piazza, Rouge-Pont, et al., 1991). Together, these data support

an integrative role for glucocorticoid function and dopamine in mediating individual differences in reward-related behaviors.

Glucocorticoids in the propensity to attribute incentive salience

The effects of stress on the propensity to attribute incentive salience to reward cues

Given the overlapping brain structures and circuits (Figure 1.1), it is perhaps not surprising that stress has been shown to impact the propensity to attribute incentive salience to reward cues (i.e. sign-tracking). Specifically, it appears that early life stress sets the stage for enhanced sign-tracking behavior in adulthood. For example, inadequate early-life social experience, consisting of artificial-rearing (i.e. deprived of mother and litter) from postnatal day (PND) 5, increases sign-tracking behavior in adulthood (PND 90-160) (Lomanowska et al., 2011). Notably, rearing in an enriched environment reduces this tendency towards sign-tracking (Beckmann & Bardo, 2012). Further, early life adversity, consisting of isolation, forced swim, restraint, and exposure to predator odor from PND 21-35, potentiates sign-tracking behavior in adulthood (PND 60+) (Hynes et al., 2018). The mechanisms by which early life stress affects the propensity to attribute incentive salience to reward cues remains to be determined. It is likely, however, that the impact of early life stress on brain development and connectivity in adulthood (Chen & Baram, 2016) renders one more likely to sign-track. Indeed, such experiences are known to alter stress responsivity and response to drugs of abuse in adulthood via alterations in brain function (Ladd et al., 2004; Sinha, 2001). Specifically, exposure to stress early in life has been reported to alter hippocampal glucocorticoid receptor (Type I and II) expression in adulthood and increase stress-induced corticosterone response (Brunton & Russell, 2010; C. Henry et al., 1994; Ladd

et al., 2004). Early life stress also results in a decrease in dopamine transporter expression and an increase in stress-induced dopamine activity (Meaney et al., 2002). We speculate, therefore, that the impact of early life stress on GC function and dopamine activity may alter the way in which these two systems interact and, in turn, promote DA-dependent stimulus-response learning, or sign-tracking behavior.

Importantly, exposure to stress later in life, seems to attenuate the attribution of incentive salience to reward cues. A single prolonged stressor, consisting of restraint, forced swim, and ether exposure in early adulthood, attenuates the acquisition of sign-tracking behavior (Fitzpatrick et al., 2019). These same rats show an attenuation of stimulated dopamine release within the nucleus accumbens. Thus, while the dopamine system appears to be a primary point of intersection for stress-induced effects on sign-tracking behavior, such effects are dependent on the type of stress and timing of exposure, with differential impacts early vs. later in life.

The role of glucocorticoids on the propensity to attribute incentive salience to reward cues

Although limited, there are also data to support a specific role for GC function in incentive learning processes. It has been shown that a single session of Pavlovian conditioning – consisting of lever-CS presentation paired with food-US delivery – elicits a rise in corticosterone in all rats, but to a greater extent in those that become sign-trackers (Flagel et al., 2009). Thus, individual differences in corticosterone response are apparent even before a goal-tracking or sign-tracking conditioned response is established. Further, systemic administration of a GR antagonist decreases sign-tracking behavior in Japanese quail (Rice et al., 2018) and does so in a dose-dependent

manner (Rice et al., 2019). Together, these data highlight a potential role for glucocorticoids in determining individual differences in the propensity to attribute incentive salience to reward cues.

Conclusion

Within the brain, GC and GR function and are nicely situated within regions that overlap or communicate with those implicated in goal- vs. sign-tracking behaviors (Figure 1.1). Their central role in response to stress and implications in multiple psychopathologies like depression, schizophrenia, and PTSD may suggest they are common indicators of aberrant behavior and potentially relevant to comorbidity. The influence of this hormone on learning and memory and the associated neuroplastic changes (e.g., LTP) suggest that glucocorticoids are important in acquiring information about the environment (e.g., cues) and recalling how to respond advantageously (e.g., a conditioned response). Further, glucocorticoids are thought to potentiate drug-related behaviors and to contribute to individual differences in such behaviors by interacting with dopamine (DA). Currently, however, our understanding of the role of glucocorticoids in positive valence systems and, in particular, in incentive motivational processes, is limited. In this regard, this dissertation includes a series of studies that have: investigated behavioral and neuroendocrine measure of negative valence in goal- and sign-tracking rats (Chapter 2), the effect of systemic GCs on the acquisition and expression of sign- and goal-tracking behaviors (Chapter 3), and GC and DA levels within the nucleus accumbens in goal- and sign-tracking rats prior to and following the acquisition of a conditioned response (Chapter 4).

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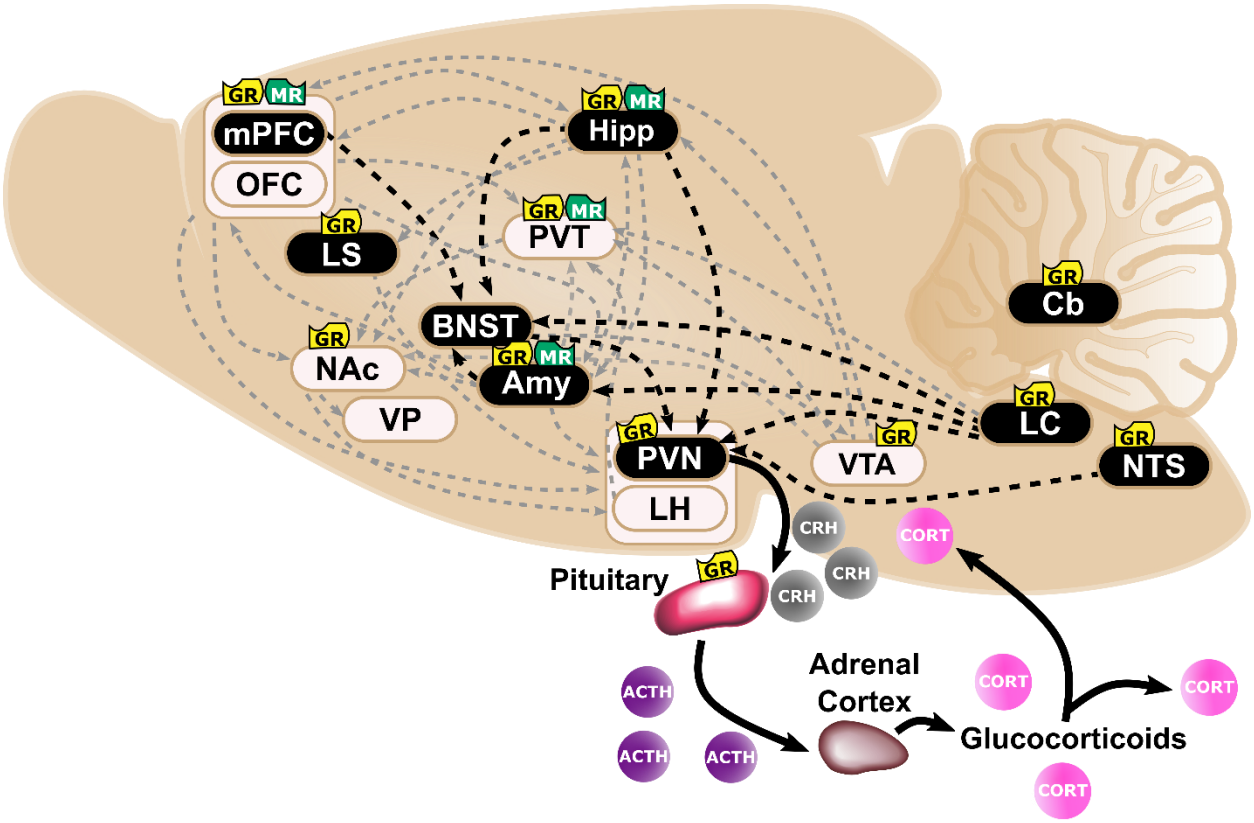

Figure 1.1 Overlapping neural circuits mediating HPA-axis "stress responsivity" and individual differences in cue-motivated behaviors. The key elements mediating the hypothalamic-pituitary-adrenal (HPA)-axis "stress response" are depicted (in black), and start with the conversion of neural information from the prefrontal cortex (PFC), hippocampus (Hipp), amygdala (Amy), and brainstem at the paraventricular nucleus (PVN) of the hypothalamus. Information is relayed directly to the PVN or indirectly via the bed nucleus of the stria terminalis (BNST). The HPA axis is triggered by the release of corticotropin-releasing hormone (CRH) from the PVN. Consequently, ACTH secretion from the pituitary is elicited; and, subsequently, the synthesis and secretion of glucocorticoids from the adrenal cortex occurs. Glucocorticoids are diffused across the body and brain. At baseline levels, they exert their effects upon type I-MRs (in green) located primarily within the hippocampus (Hipp) and less so within the PFC, Amy, and paraventricular nucleus of the thalamus (PVT). Under stress or circadian peak, they activate Type II GRs (in yellow) located ubiquitously across the pituitary, PVN, PVT, Amy, PFC, Hipp, lateral septum (LS), nucleus accumbens (NAc), ventral tegmental area (VTA), nucleus tractus solitarus (NTS), locus coeruleus (LC), and cerebellum (Cb). (Ahima & Harlan, 1990; Chao et al., 1989; Fuxe, Harfstrand, et al., 1985; Fuxe, Wikstrom, et al., 1985; Jaferi & Bhatnagar, 2006; Reul & de Kloet, 1985). Structures that comprise the "motive circuit" (Kalivas & Volkow, 2005; Kelley et al., 2005) and have been implicated in individual differences in cue-learning (Flagel & Robinson, 2017) are shown in light beige. This includes: medial PFC(mPFC) (Campus et al., 2019; Haight et al., 2017), orbitofrontal cortex (OFC) (Stringfield et al., 2017), Hipp (Fitzpatrick et al., 2016), NAc (Saunders & Robinson, 2012), ventral pallidum (VP) (Ahrens et al., 2016), Amy (Flagel et al., 2011), lateral hypothalamus (LH) (Haight et al., 2020; Haight et al., 2017), PVT (Campus et al., 2019), and VTA (Flagel et al., 2011)

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Chapter 2

Neuroendocrine and Behavioral Measures of Negative Valence in Male Goal-tracker and Sign-tracker Rats

Note: The text, and figures, within Chapter 2 have appeared previously in a pre-print (Lopez et al., 2020, under review in eNeuro), and are reproduced here with permission from the authors.

Abstract

Cues, or stimuli in the environment, attain the ability to guide behavior via learned associations. As predictors, cues can elicit adaptive behavior and lead to valuable resources (e.g., food). For some individuals, however, cues are transformed into incentive stimuli and can elicit motivational states that can be maladaptive. The goal-tracker/sign-tracker animal model captures individual differences in cue-motivated behaviors, with reward-associated cues serving as predictors of reward for both goal-trackers and sign-trackers, but becoming incentive stimuli to a greater degree in sign-trackers. While these distinct phenotypes are characterized based on Pavlovian conditioned approach behavior, they exhibit differences on a number of behaviors of relevance to psychopathology. To further characterize the neurobehavioral endophenotype associated with individual differences in cue-reward learning, neuroendocrine and behavioral profiles associated with negative valence were investigated in male goal-tracker, sign-tracker, and intermediate responder rats.

It was revealed that baseline corticosterone increases with Pavlovian learning, and that this increase is positively associated with the development of sign-tracking. No significant differences were observed between goal-trackers and sign-trackers in behavior during an elevated plus maze or open field test, nor differences in the corticosterone response to the open field test or physiological restraint. However, it was demonstrated that sign-trackers have greater glucocorticoid receptor mRNA expression in the ventral hippocampus, with no phenotypic differences in the dorsal hippocampus. These findings suggest that goal-trackers and sign-trackers do not differ on tests that have negative valence; rather, differences in neuroendocrine measures between these phenotypes can be attributed to distinct cue-reward learning styles**.**

Introduction

Through learned associations, environmental cues become predictors of biologically relevant stimuli. In turn, such cues elicit an adaptive response, facilitating behavior towards valuable resources. However, for some individuals, cues elicit complex emotional responses and motivational states that may prompt adaptive, but also maladaptive behavior. For example, upon exposure to drug-associated cues, individuals with addiction report drug-craving and, consequently, often relapse (Ehrman et al., 1992). Similarly, when exposed to trauma-related stimuli, individuals with post-traumatic stress disorder (PTSD) report hyperarousal and anxiety (Shin et al., 2004). Cues attain the ability to elicit extreme emotional states and aberrant behavior when they are attributed with excessive incentive motivational value, or incentive salience (Robinson & Berridge, 1993). The propensity to attribute incentive salience to

environmental cues, thereby, may reflect a vulnerability trait for cue-motivated psychopathologies, like addiction and PTSD (Flagel et al., 2010; Morrow et al., 2011).

Individual variation in the propensity to attribute incentive salience to reward cues can be captured using a Pavlovian conditioned approach (PavCA) paradigm, consisting of a lever-cue paired with delivery of a food-reward (Robinson & Flagel, 2009). Upon lever-cue presentation, goal-trackers (GTs) direct their behavior towards the location of reward delivery, whereas sign-trackers (STs) direct their behavior towards the cue itself. For both GTs and STs the cue attains predictive value, but for STs the cue also attains incentive value and is transformed into a “motivational magnet” (Berridge et al., 2009). Intermediate responders (IRs) vacillate between goal- and cue-directed behavior, without an innate preference for either cue-learning strategy. GTs and STs differ on a number of traits of relevance to psychopathology. Relative to GTs, STs are more impulsive (Lovic et al., 2011), exhibit an exaggerated fear response to aversive stimuli (Morrow et al., 2011; Morrow et al., 2015), show poor attentional control (Paolone et al., 2013), and have a greater propensity for reinstatement of drug-seeking behavior (Flagel et al., 2010; Saunders & Robinson, 2010, 2011; Saunders et al., 2013; Yager & Robinson, 2013, also see Kawa et al., 2016). These behavioral phenotypes are subserved by distinct neural mechanisms (Campus et al., 2019; Flagel, Clark, et al., 2011; Pitchers et al., 2017). While GTs seem to rely on “top-down” cortical control, STs are presumed to be driven by subcortical “bottom-up” circuitry (Flagel & Robinson, 2017; Kuhn et al., 2018; Sarter & Phillips, 2018). Thus, the GT/ST model captures a neurobehavioral endophenotype reflective of more than individual differences in cue-reward learning.

Most of the research surrounding the GT/ST model has focused on “positive valence” and the associated neurobiology, and only a few studies have investigated indices of “negative valence” (e.g., Harb & Almeida, 2014; Morrow et al., 2011; Vanhille et al., 2015). The Research Domain Criteria (RDoc), put forth by the National Institute of Mental Health, defines “positive valence systems” as those responsible for responses to motivational situations, including reward learning; and “negative valence systems” as those responsible for responses to aversive situations, including fear and anxiety (RDoC). Per the RDoC, corticosterone (CORT), the final product of the hypothalamic-pituitary-adrenal (HPA) axis in rodents, is a molecular marker of negative valence. However, we know that the role of CORT extends beyond the “stress response” and into arenas of learning and memory (e.g., Sandi et al., 1997), reward-learning (e.g., Tomie et al., 2002), and reinforcement (e.g., Piazza et al., 1993). Of particular relevance, CORT is involved in forming Pavlovian associations for both aversive (e.g., Marchand et al., 2007) and appetitive (e.g., Tomie et al., 2002) stimuli (for review, see Lopez & Flagel, 2020). With respect to the latter, relative to GTs, STs show a greater rise in CORT following an initial PavCA session, prior to the development of a conditioned response (Flagel et al., 2009). In the current study, the role of CORT in the propensity to attribute incentive value to reward cues was assessed by examining baseline levels before and after the acquisition of PavCA behavior (Experiment 1A). In addition, negative valence systems were probed within the context of the GT/ST animal model and behavioral and CORT responses to environmental challenges were assessed, including the elevated plus maze, open field test, and acute physiological restraint (Experiment 1B). As the hippocampus is a central component of negative

valence circuits (RDoC), and glucocorticoid receptors (GRs) within the hippocampus are key regulators of HPA-axis activity (Akil, 2005; Herman et al., 1989), hippocampal GR mRNA was assessed in GTs, STs, and IRs (Experiment 2). These studies allowed us to expand our characterization of the neurobehavioral endophenotype captured by the GT/ST model, and to begin to deconstruct the role of CORT beyond negative valence systems.

Materials and Methods

Experiment 1: General procedures

Animals

For Experiments 1A and 1B, sixty male Sprague-Dawley rats were obtained from Charles River Breeding Labs (Colony C72 Saint-Constant, Canada, and Colony R04 Raleigh, NC, USA). Rats weighed between 225-275 g upon arrival and were pair-housed in standard acrylic cages (46 x 24 x 22 cm) in a temperature-controlled room (22 ± 2°C) under a 12-h light/dark cycle (lights on at 7:00). Food and water were available ad libitum for the duration of the study. Rats were allowed to acclimate to their colony room and remained undisturbed in their homecages for seven days after arrival. Rats were then briefly handled every day for five consecutive days before any experimental manipulation. During the last two days of handling, twenty-five 45-mg banana-flavored grain pellets (Bio-Serv, Flemington, NJ, USA) were placed inside the homecage, allowing rats to habituate to the food reward used during Pavlovian conditioned approach (PavCA) training. Behavioral testing occurred during the light cycle (between 10:00 and 14:00). All experimental procedures followed The Guide for

the Care and Use of Laboratory Animals: Eight Edition (2011, National Academy of Sciences).

Behavioral testing

Pavlovian conditioned approach (PavCA) training

All PavCA training took place in standard behavioral testing chambers (MED Associates, St. Albans, VT, USA; 20.5 × 24.1 cm floor area, 29.2 cm high) located inside a room with red lighting. The chambers were enclosed in sound-attenuating boxes equipped with a ventilation fan that provided constant air circulation and served as white noise. Each chamber contained a food-cup centered on one of the walls and placed 6 cm above the grid floor. The food-cup was equipped with an infrared beam, and each beam break was recorded as a head entry. Counterbalanced, right or left of the food-cup, was a retractable lever that illuminated upon presentation and was also placed 6 cm above the floor. A force of at least 10 g was necessary to deflect the lever; this deflection was recorded as a "lever contact." On the opposite wall, a white house light was placed 1 cm from the top of the chamber. House light illumination signaled the beginning of the session and remained on for the duration of the session.

Rats underwent a single pre-training session, where the food-cup was baited with three-grain pellets in order to direct the rat’s attention to the location of the reward. Once placed in the chamber, the house light turned on after 5 min, signaling the beginning of the session. The pre-training session consisted of 25 trials during which the lever remained retracted, and pellets were delivered randomly into the food-cup; one pellet per trial on a variable interval 30 s schedule (range 0-60 s). The total session length was approximately 12.5 min.

Following pre-training, or twenty-four hours later, rats underwent a total of five consecutive PavCA training sessions. Each session consisted of 25 trials on a variable interval 90 s schedule (VI 90, range 30-150 s) during which an illuminated lever (conditioned stimulus, CS) was presented for a total of 8 s, and immediately upon its retraction, a food pellet (unconditioned stimulus, US) was delivered into the adjacent food-cup. Each session lasted approximately 40 min.

The following behavioral measures were recorded during each PavCA session: (1) number of lever contacts, (2) latency to contact the lever for the first time, (3) probability to contact the lever, (4) number of food-cup entries during presentation of the lever, (5) latency to first enter the food-cup during presentation of the lever, (6) probability of entering the food-cup during presentation of the lever, and (7) number of food-cup entries during the inter-trial interval. These values were then used to calculate three measures of approach behavior that comprise the PavCA index: (1) response bias [(total lever presses – total food-cup entries) ÷ (total lever presses + total food-cup entries)], (2) probability difference [probability to approach the lever – the probability to enter the food-cup], (3) latency difference [± (latency to approach the lever – latency to enter the food-cup) ÷ 8]. As previously described (Meyer et al., 2012), PavCA index score was calculated from the average of sessions 4 and 5 using this formula: [(response bias + probability difference + latency difference) ÷ 3]. Scores ranged from +1 to -1; a more positive score indicated a preference for sign-tracking behavior and a negative score for goal-tracking. The cutoffs for phenotype classification were: ≤ -0.5 for a GT, ≥ 0.5 for a ST, and in between -0.5 and 0.5 for an IR, those that vacillate between the two conditioned responses.

Corticosterone

Sample collection

To investigate plasma corticosterone (CORT) profiles, blood samples were collected via lateral tail nick at the time points indicated below for Experiment 1A and 1B (see also Figure 2.1A). An experimenter lightly restrained each rat under a blue pad near the edge of a flat surface, allowing their tail to hang off. A small (≤ 5 mm) nick was made by a second experimenter with the tip of a razor blade, and blood was extracted via capillary action (~200 µL) into an EDTA-coated tube (Sarstedt, Nümbrecht, Germany). Samples were capped, inverted 2-3 times, and immediately placed onto ice where they remained until the last tail nick was performed (< 3 hr standing time). Samples were then separated by centrifugation (13,000 rpm for 10 min at 4 ºC), and plasma was extracted, flash-frozen on dry ice, and stored at -20 ºC until processed for radioimmunoassay.

Radioimmunoassay

Plasma CORT levels were measured using commercially available CORT I^{125} Double Antibody Radioimmunoassay (RIA) kit (MP Biomedicals, Solon, OH) with a minimum detectable dose of 7.7 ng/mL. The manufacturer's protocol was followed verbatim. A range of 25-1000 ng/mL CORT calibrators was used to generate a standard logarithmic curve for every set of 76 test tubes (the centrifuge test tube capacity for one spin). For Experiments 1A and 1B, a total of 482 plasma samples (not including duplicates or calibration standards) were assayed using 19 centrifuge spins across 6 days, with no more than 4 sets (i.e., centrifuge spins) per day. Gamma radiation counts per minute were averaged across duplicate samples and converted into CORT

concentrations using the average standard curve generated from all sets that were run for each day of RIA. On average, the intra-assay coefficient of variation was 7.24%, while the inter-assay coefficient of variation was 16.44%. Outliers were identified and removed if: 1) duplicates had a percent error greater than 10%, or 2) samples were identified as an extreme outlier (3x the interquartile range) by statistical software, or 3) notes confirmed an observable reaction to tail nicks (e.g., vocalization).

Experiment 1A: Pavlovian conditioned approach behavior and baseline plasma corticosterone profiles

Corticosterone

Sample collection

Samples were collected, as described above, under baseline conditions before Pavlovian conditioned approach training (Pre-PavCA) and following the development of a conditioned response to the lever-cue (Post-PavCA) (refer to the experimental timeline, Figure 2.1A). Pre-PavCA tail nicks were performed 24 h prior to the pre-training sessions (see Experiment 1 Behavioral Testing), while Post-PavCA tail nicks were performed 24 h after the last session (Session 5) of training. On days of collection, six rats were transported in their paired-housed homecages into a designated room (start 10:30), where all collection took place under white light. Tail nicks were performed one at a time (~ 60-90 s per collection). Each wave of six rats remained in the room together but on the opposite side from the collection area. After the last tail nick was performed, all rats were promptly returned to the colony room. Rats were left undisturbed for a total of ten-days before Experiment 1B began.

Experiment 1B: Behavioral and corticosterone response to tests of negative valence in goal-trackers, sign-trackers, and intermediate responders

Corticosterone

Sample collection

Plasma CORT levels induced by behavioral assays of negative valence and physiological restraint were captured using tail-nick sampling procedures as described above. Collections took place 24 h before the open field test (time 0, or baseline) and 20, 40, 60, and 80 min post-onset of the test. For restraint-induced CORT profiles, collections took place immediately when rats were placed into the restraining device (time 0, or baseline) and 30 (before being released), 60, 90, and 120 min after the onset of restraint. Rats were transported into the designated room in a staggered fashion, one at a time to begin collections. Repeated nicks were performed on each rat to capture all of the time points. Up to 9 rats remained together in the designated collection room but were on the opposite side of the room from the collection area. Rats were returned to the colony room in a staggered fashion after their last sample was collected.

Behavioral testing

Elevated plus maze (EPM)

After a ten-day rest period following Experiment 1A, rats were exposed to an elevated plus maze (EPM) test (refer to experimental timeline, Figure 2.1A), considered to be a metric of anxiety-like and risk-taking behavior (Lister, 1987; Walf & Frye, 2007). The apparatus was constructed of four connected arms (each 70 cm from the floor, 45 cm long, and 12 cm wide) made of black Plexiglass and arranged in a cross shape. 45-cm high walls enclosed two opposite arms, and the remaining two were open platforms.

A central square (12 x 12 cm) connected all four arms. The test room was dimly lit (40 lux) by a light fixture located above the maze. Prior to the test, rats were transported inside their homecage, along with their cage mate, into the testing room and left undisturbed to acclimate for 30 min. Upon starting the test, each rat was placed in the central platform facing an open arm and allowed to roam freely around the maze for a total of 5 min. The experimenter remained in the room but was distanced from the apparatus in order to be out of the rat's view. A video-tracking system (Noldus Ethovision 11.5, Leesburg, VA) using a live feed from a digital camera mounted on the ceiling directly above the center of the maze was used to detect and record: 1) latency to enter the open arms for the first time, 2) frequency to enter each arm, and 3) time spent in each arm. Additionally, universally used risk assessment behaviors (RABs, see Mikics et al., 2005; Rodgers et al., 1999) were scored manually by the experimenter viewing the live recording. Specifically, the number of times the rat exhibited a bout of grooming, rearing, and protected and unprotected head dips (i.e., head dips over the side of the maze while their body was inside an enclosed arm vs. their body being completely exposed on the open platforms) was quantified.

Open field test (OFT)

After a ten-day rest period following EPM testing, rats were exposed to an open field test (OFT; refer to the experimental timeline, Figure 1A), a metric of negative valence (see RDoC) and exploratory behavior (Walsh & Cummins, 1976). The OFT test occurred in the same room as the EPM test, and again, paired-housed rats were transported from the colony room to the dimly lit test room and allowed to acclimate for ~30 min before testing began. The open field apparatus was a 4-wall Plexiglass

enclosure with an open top and plexiglass floor (100 x 100 x 50 cm). At the start of the test, rats were placed into the same corner (bottom left) of the arena and allowed to roam freely for 5 min. Behavior was video recorded with a digital camera mounted above the apparatus. Noldus Ethovision (11.5, Leesburg, VA) was used to detect: 1) the time spent in the center of the arena (a 50 x 50 cm square drawn in the center), 2) the time spent in the outer edge of the arena (25 cm wide border), 2) the number of entries into the center arena, 3) latency to enter the center of the arena for the first time, and 4) total distance traveled.

Restraint

After a ten-day rest period following the OFT, rats underwent a single session of physiological restraint. The restraining device consisted of a white 9 x 12 cm sleeve of flexible Teflon secured with two black Velcro straps attached to a 9 x 3 cm clear Plexiglas platform with a tail slit on one end and breathing holes on the other. Rats were transported in their homecage into the testing room, which was the same as that used for Experiment 1A and OFT time-course measures. Rats were placed into the restrainer and remained there for 30 min.

Experiment 2: Glucocorticoid receptor (GR) mRNA expression within the hippocampus of goal-trackers, sign-trackers, and intermediate responders

Animals

An additional sixty male Sprague-Dawley rats were obtained from Charles River Breeding Labs (C72 and R04) for this experiment. Housing and testing conditions were identical to those described in Experiment 1, except that lights turned on and off at 6:00

and 18:00 h, respectively. Rats were exposed to 2 days of handling prior to behavioral testing, which occurred between 11:00 and 15:00 h.

Behavioral testing

Pavlovian conditioning approach (PavCA) training

PavCA training and classification of GT, ST, and IR were performed identically to that described above for Experiment 1.

Glucocorticoid receptor mRNA expression

Tissue collection

Twenty-four hours after completion of the 5th PavCA training session (refer to the experimental timeline, Figure 2.1B), rats underwent live decapitation, and their brains were extracted and immediately flash frozen in 2-methyl butane (-30ºC). Brains were stored at -80ºC until further processing. Frozen brains were mounted perpendicularly to a metal cryostat chuck using Optimal Cutting Temperature compound (Fisher Healthcare, Thermo Fisher Scientific Kalamazoo, MI, USA) and coated with Shandon M-1 embedding matrix (Thermo Fisher Scientific, Kalamazoo, MI, USA) in preparation for sectioning. Whole brains were coronally sectioned at 10 µm on a cryostat at -20°C. Brain sections were collected, 4.68 to -7.08 mm from Bregma, and directly mounted onto Superfrost Plus microscope slides (Fischer Scientific, Pittsburg, PA, USA), with four sections per slide and ~200 µm between sections on a given slide. Slides were stored at -80°C in preparation for in situ hybridization.

Probe synthesis

Probes for in situ hybridization were synthesized in-house using rat mRNA sequences complementary to the RefSeq database number (M14053) for Type II

glucocorticoid receptor (GR: insert size 402, insert location nucleotides 765-1167) (same as Garcia-Fuster et al., 2012). All cDNA segments were extracted using a Qiaquick Gel Extraction kit (Qiagen, Valencia, CA), subcloned in Bluescript SK (Stratagene, La Jolla, CA), and confirmed by nucleotide sequencing. The probes were labeled in a reaction mixture of 2µl of linearized DNA specific to the probe, 10X transcription buffer (Epicentre Technologies Madison, WI), 3 µL of S-35-labeled UTP, 10 µL of S-35-labeled ATP, 1 µL of 10 mM CTP and GTP, 1µL of 0.1M dithiothreitol (DTT), 1µl of RNAse inhibitor, and 1µl of T7 RNA polymerase and incubated for 1.5 hours (37°C). Labeled probes were then purified using Micro Bio-Spin 6 Chromatography Column (BioRad, Berkeley, CA), and 1 µl of the probe was counted for subsequent radioactivity dilution calculations. Four to six labelings were used to reach the necessary volume and optimal radioactivity (1-2 million counts per minute/ slide). An additional 1µl of 1M DTT was also added to the labeled mRNA after purification, allowed to incubate at room temperature for 15 min, and stored at -20°C until further use.

In situ hybridization

The radioactive probe was diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 3X saline-sodium citrate buffer, 50 mM sodium phosphate buffer, 1X Denhardt's solution, 0.1 mg/ml yeast tRNA, and 10 mM DTT) and the volume calculated based on the initial count in order to obtain roughly 1-2 x 10^6 radioactivity counts per 75 µL of the diluted probe in hybridization buffer. Slide-mounted brain tissue, -1.08 to -7.08 mm from Bregma, was fixed in 4% paraformaldehyde solution (1 hr), washed in 2X saline-sodium citrate buffer (SSC), and incubated with 0.1 M triethanolamine (TEA) with 0.25% acetic anhydride (10 min). Slides were then dehydrated using ascending ethanol

concentrations and air-dried for 1 hour. Hybridization buffer was warmed (37°C) and mixed with the calculated quantity of probe and 1 M DTT (~1% total HB volume). 75 µl of the diluted probe was then applied to coverslips, which were subsequently placed onto the tissue. Slides were then placed in humidity-maintained hybridization chambers soaked with formamide and incubated overnight (~16 hrs) at 55°C. The next day, coverslips were removed, and the slides were rinsed with 2X SSC. Slides were then incubated (1 hr) in RNase A solution (100 µg/mL RNase in Tris buffer with 0.5M NaCl, 37°C), washed in descending concentrations of SSC (2X, 1X, 0.5X), and incubated (1 hr) in 0.1X SSC (65°C). Next, sections were briefly rinsed in H_2O, dehydrated using ascending ethanol concentrations, and air-dried for 1 hr. Slides were then loaded into film cassettes and exposed in a dark room to 35 x 43cm Kodak BioMax MR film (Carestream Health Inc, Rochester, NY, USA) for seven weeks. Extra slides using spare experimental tissue were run concurrently to confirm optimal exposure time. The specificity of the probe was verified using sense strand controls similar to previous studies (Garcia-Fuster et al., 2012; Kabbaj et al., 2000).

Quantification

Films were developed using Microtek ScanMaker 1000XL (Fontana, CA, USA) and digitally scanned using SilverFast Lasersoft Imaging software (Sarasota, FL, USA). Signal expression was quantified using ImageJ (National Institutes of Health, Bethesda, MD), a computer-assisted optical densitometry software. The brush selection tool (size: 15 pixels) was used to trace the subregions of interest (CA1, CA2, CA3, and dentate gyrus) throughout the dorsal hippocampus (-2.64 to -4.56 mm from Bregma) and ventral hippocampus (-4.68 to -6.72 mm from Bregma), using the Rat Brain Atlas (Paxinos and

Watson, 2007) for guidance. Area (total number of pixels), optical density (darkness of pixels) and integrated optical density (intensity and spread) measurements of the region of interest were taken using a macro that automatically enabled signal above background (3.5 x standard deviation) to be determined. The area (unit, 63 pixels/ 1 mm) and optical density (darkness) were calculated for each of the four hippocampal subregions across a range of 11-21 sections per rat that spanned the dorsal-ventral gradient of the hippocampus. A single value was calculated for each of the hippocampal subregions per rat, by averaging the values of both hemispheres across multiple sections. Further, given that the dorsal and ventral hippocampus are viewed as neuroanatomically and functionally distinct (see Fanselow & Dong, 2010), with the dorsal hippocampus considered to be more involved in cognitive function and the ventral hippocampus in stress and emotion, a single average value was used for dorsal vs. ventral subregions and data were graphed and analyzed separately (similar to Romeo et al., 2008). Sections with damaged tissue or unusual signal (e.g., dark artifacts) that distorted the region of interest were omitted from analyses. During quantification, the experimenter was blind to the phenotypes of each subject.

Experiments 1 and 2: Statistical analysis

Behavioral outcome measures (i.e., PavCA, EPM, OFT), plasma corticosterone concentrations, and in situ hybridization measures (i.e., area and optical density) were analyzed using the Statistical Package for the Social Sciences (SPSS) program version 24.0 (IBM, Armok, NY, USA). Linear mixed-effects models were performed for PavCA behavior and neuroendocrine measures (corticosterone and GR mRNA levels), using the best fit covariance structure with the lowest Akaike's information criterion for each

set of data. Univariate analysis of variance was performed for EPM and OFT behavior and tested for normality using the Shapiro-Wilk test. When dependent variables failed to meet normality, log 10 or square root transformations were conducted, or a Kruskal-Wallis nonparametric test was performed (using StatView, version 5.0, SAS Institute Inc., Cary, NC, USA). Pearson correlations were performed to determine if there was a significant correlation between baseline CORT levels (pre- vs. post-PavCA) and baseline CORT levels and PavCA behavior. Statistical significance was set at $p<0.05$, and Bonferroni post-hoc analyses were conducted when significant interactions were detected. All figures were made using GraphPad Prism 7.

Results

Experiment 1: Pavlovian conditioned approach behavior and baseline plasma corticosterone profiles

PavCA behavior

The following lever-directed (sign-tracking) and food cup-directed (goal-tracking) behaviors were assessed across five consecutive PavCA training sessions and compared between GTs (n=11), IRs (n=17), and STs (n=32): the probability to approach, the number of contacts, and the latency to approach the lever or food-cup during the presentation of the lever-CS (Figure 2.2). Main Effects of Phenotype, Session, and Phenotype x Session interactions for all behavioral measures are reported in Table 2.1 (top). There was a significant Effect of Phenotype and Session for all behavioral measures. As expected, STs showed a significantly greater probability to approach the lever (Figure 2.2A), a greater number of lever contacts (Figure 2.2C), and shorter latency to approach the lever (Figure 2.2E), relative to IRs and GTs. These

differences in lever-directed behaviors were apparent by the 2nd PavCA training session (Figure 2.2, also Table 2.2 (top left)). In contrast, relative to STs and IRs, GTs showed a significantly greater probability of approaching the food-cup (Figure 2.2B), a greater number of food-cup entries (Figure 2.2D), and a shorter latency to enter the food-cup (Figure 2.2F). These differences in food cup-directed behavior became apparent by the 3rd PavCA training session (Figure 2.2, also Table 2.2 (top right)).

Baseline CORT levels pre- and post-PavCA

CORT levels

Overall, pre- and post-PavCA baseline plasma CORT levels did not significantly differ between Phenotypes (GTs n=11, IRs n=13, STs n=14) [Effect of Phenotype: $F_{(2,57.691)}$=2.325, p=0.107] (Figure 2.3A). Relative to pre-PavCA, post-PavCA baseline CORT levels were significantly higher [Effect of Timepoint: $F_{(1,53.246)}$=20.180, p<0.001], rising from an overall average of 56 ng/mL (pre-PavCA) to 108 ng/mL (post-PavCA). While, baseline CORT levels appear to rise with the experience of PavCA training, the extent to which CORT increased was not dependent on Phenotype [Time-point x Phenotype interaction: $F_{(2, 52.633)}$=0.535, p=0.589]. These data are in agreement with prior studies (Flagel et al., 2009; Tomie et al., 2000), demonstrating that pre-PavCA baseline plasma CORT levels do not significantly differ between Phenotypes, and extend these findings to show that baseline plasma CORT levels also do not differ between Phenotypes after the development of a conditioned response.

Correlations

To further investigate the relationship between baseline CORT levels and cue-motivated behavior, we performed correlational analyses. Pre-PavCA baseline levels

predict post-PavCA baseline levels [r= 0.45, *p*=0.001], and the correlation coefficient is greater when STs are analyzed separately [r=0.56, *p*=0.002] (Figure 2.3B). Although pre-PavCA baseline levels do not appear to be predictive of the behavioral phenotype that emerges with PavCA training (i.e., the average PavCA index from session 4 and 5) [r= 0.18, *p*=0.198] (data not shown), post-PavCA baseline CORT levels are significantly correlated with the magnitude of change in the conditioned response from the onset of training (Session 1) to the end of training (Session 5) [r= 0.29, *p*= 0.04]. That is, a shift toward a more positive PavCA index (i.e., development of a stronger sign-tracking response) correlates with higher post-PavCA baseline CORT levels; and, again, this relationship is more apparent when STs are considered independently [r=0.44, *p*= 0.02] (Figure 2.3C).

Experiment 1B: Behavioral and corticosterone response to tests with negative valence in goal-trackers, sign-trackers, and intermediate responders

Elevated Plus Maze

GTs (n=11) and STs (n=14) did not significantly differ on any behavioral outcome measure of the EPM test, but statistical analysis revealed significant differences relative to their intermediate responder counterparts (n=13). While all rats spent the most time [Effect of Zone: $F_{(2,105)}$=140.397, *p*<0.001] inside the closed arms ($\bar{x}$=51.21%), relative to the open arms ($\bar{x}$=21.91%) or center square ($\bar{x}$=26.88%), IRs spent significantly less time [Phenotype x Zone interaction: $F_{(4,105)}$=2.762, *p*=0.031] inside the open arms, relative to GTs (*p*=0.011) (Figure 2.4). However, the latency to enter the open arms for the first time was similar across all Phenotypes [Effect of Phenotype: $F_{(2,35)}$=0.187, *p*=0.83] (data not shown) and, in general, relative to IRs, the extreme Phenotypes (GTs,

p=0.02, and STs, *p*=0.049) entered different zones of the EPM more frequently [Effect of Phenotype: $F_{(2,105)}$=6.744, *p*=0.002] (data not shown). Additionally, there were no significant differences between Phenotypes for any of the risk assessment behaviors during the EPM test: frequency of grooming [Kruskal-Wallis test, Effect of Phenotype: $\chi^2_{(2)}$=1.984, *p*=0.371], rearing [Effect of Phenotype: $F_{(2,35)}$=2.232, *p*= 0.122], protected head dips [Effect of Phenotype: $F_{(2,35)}$=0.496, *p*=0.613], or unprotected head dips [Effect of Phenotype: $F_{(2,24)}$=0.207, *p*=0.814] (data not shown).

Open field test

There were no significant differences between Phenotypes in their behavior on the OFT. All rats spent a comparable amount of time in the outer edge of the arena [Kruskal-Wallis, Effect of Phenotype: $\chi^2_{(2)}$=2.012, *p*=0.366] (Figure 2.5). There were no significant differences in the number of entries to the center of the arena [Kruskal-Wallis Effect of Phenotype: $\chi^2_{(2)}$=3.029, *p*=0.220] (data not shown), latency to enter the center of the arena [Kruskal-Wallis Effect of Phenotype: $\chi^2_{(2)}$=2.345, *p*=0.310] (data not shown), or time spent in the center of the arena [Kruskal-Wallis, Effect of Phenotype: $\chi^2_{(2)}$=2.053, *p*=0.358] (Figure 2.5). The distance traveled during the OFT was also similar between phenotypes [Kruskal-Wallis Effect of Phenotype: $\chi^2_{(2)}$=3.287, *p*=0.193] (data not shown). Consistent with data from the EPM, STs, GTs, and IRs do not exhibit differences in behavioral tests of negative valence.

Corticosterone response

Corticosterone response to OFT

Exposure to the OFT elicited a CORT response [Effect of Time: $F_{(4,44.652)}$=12.849, *p*<0.001], with a significant rise relative to baseline at 20, 40, 60, and 80 min post-OFT

onset. Although the CORT response was decreased at 80-min relative to the peak response (40 vs. 80 min, $p<0.001$), a return to baseline levels was not captured with this time course (baseline vs. 80 min, $p= 0.041$). Nonetheless, the CORT response to the OFT did not significantly differ between phenotypes [Effect of Phenotype: $F_{(2, 35.564)}=0.215$, $p=0.808$; Time x Phenotype interaction: $F_{(8,45.180)}=.718$, $p=.675$)] (Figure 2.6A)**.**

Corticosterone response to physiological restraint

Acute physiological restraint (30 min) elicited a CORT response [Effect of Time: $F_{(3,28.058)}=157.308$, $p<0.001$], with a significant rise relative to baseline at 30 and 90 min, and return to baseline levels at 120 min post-onset of restraint. There was not a significant difference in the CORT response to acute restraint between Phenotypes [Effect of Phenotype: $F_{(2,32.084)}=.114$, $p=0.893$; Time x Phenotype interaction: $F_{(6,29.646)}=1.568$, $p=0.191$] (Figure 2.6B).

Experiment 2: Glucocorticoid receptor (GR) mRNA expression within the hippocampus of goal-trackers, sign-trackers, and intermediate responders

PavCA behavior

Similar to Experiment 1, there were significant Effects of Phenotype, Session, and Phenotype x Session interactions for all behavioral measures reported in Table 2.1 (bottom) (data are not shown in graphical format). Differences between Phenotypes were apparent for lever- and food cup-directed behavior as early as the first PavCA training session (see Table 2.2 bottom).

GR mRNA expression

Dorsal hippocampus **–** There were no significant differences between Phenotypes in GR mRNA expression (i.e., optical density) in the dorsal hippocampus [Effect of Phenotype: $F_{(2,108)}$=0.233, *p*=0.793], and no significant difference in expression patterns between subregions of the dorsal hippocampus [Effect of Subregion: $F_{(3,108)}$=1.089, *p*=0.357] (Figure 2.7C). Given the anatomical variability in size between subregions (see schematic Figure 2.7A), significant differences in area were detected [Effect of Subregion: $F_{(3, 108)}$=1020.291, *p*<0.001] (data not shown); CA1 Subregion contained the largest area ($\bar{x}$=1.132), while CA2 the smallest ($\bar{x}$=0.147). However, the regions of interest were manually outlined (CA1, CA2, CA3, DG), and the area was not dependent on Phenotype [Effect of Phenotype: $F_{(2,108)}$=0.118, *p*=0.889; Phenotype x Subregion interaction: $F_{(6,108)}$=0.417, *p*=0.866], indicating that the selection of regions of interest was consistent across phenotypes.

Ventral hippocampus **–** Unlike the dorsal hippocampus, GR mRNA expression significantly differed between phenotypes [Effect of Phenotype: $F_{(2, 108)}$=4.601, *p*=0.012; Figure 2.7E] and subregions [Effect of Subregion: $F_{(3,108)}$=30.464, p<0.001; Figure 2.7D] in the ventral hippocampus. STs ($\bar{x}$=.497) had greater optical density relative to GTs ($\bar{x}$=.458, *p*=0.022) and IRs ($\bar{x}$=.461, *p*=0.040), and there was greater optical density in CA1 ($\bar{x}$=.563) relative to CA2 ($\bar{x}$=.441, *p*<0.001), CA3 ($\bar{x}$=.413, *p*<0.001), and DG ($\bar{x}$=.472, *p*<0.001) (Figure 2.7D). Like the dorsal hippocampus, area was significantly different between Subregions [Subregion: $F_{(3,108)}$=172.935, *p*<0.001], but not between Phenotypes [Effect of Phenotype: $F_{(2,108)}$=0.579, *p*=0.562; Phenotype x Subregion interaction: $F_{(6,108)}$=0.876, *p*=0.515] (data not shown).

Discussion

The present studies examined whether differences in behavioral and neuroendocrine measures in response to tests with negative valence are included amongst the co-existing traits associated with the propensity to attribute incentive salience to reward cues. We report three main findings. First, across phenotypes, there is a general increase in baseline plasma CORT levels over the course of associative cue-learning, and this rise is positively correlated with the development of the sign-tracking response. Second, behavioral and CORT responses to environmental challenges reflective of negative valence do not differ between goal-trackers and sign-trackers. Third, sign-trackers have greater expression of GR mRNA in the ventral hippocampus relative to goal-trackers and intermediate responders.

The basis of our understanding of CORT function in appetitive Pavlovian conditioning stems from the work of Tomie et al. (2000) who demonstrated that cue-food associations elicit an increase in plasma CORT. Subsequently, it was shown that, relative to GTs, STs exhibit a greater rise in plasma CORT following a single Pavlovian conditioning session; that is, before the development of a conditioned response (Flagel et al., 2009). Further, prior to PavCA training, baseline CORT is similar across GTs, IRs, and STs (Flagel et al., 2009); and, based on work from Tomie et al. (2000), we would not expect there to be differences between phenotypes in baseline CORT later in training. To date, however, baseline plasma CORT had not been systematically assessed in the same rat to determine if CORT profiles change as a consequence of cue-learning. Thus, in Experiment 1A we compared, within the same rat, baseline CORT concentrations at a “naïve” state of learning (pre-PavCA) and once a conditioned response had been acquired (post-PavCA). There was a significant rise in baseline

plasma CORT levels with the development of a conditioned response, and pre-PavCA CORT levels were predictive of post-PavCA CORT levels. However, CORT levels were not dependent on the innate cue-learning strategy that was employed, as CORT did not significantly differ between phenotypes. Nonetheless, to determine whether CORT serves as a predictor of cue-learning, we assessed the relationship between baseline CORT profiles and behavior. Although baseline CORT levels at either time point did not significantly correlate with behavior, we found that the change in behavior, or the rate of learning between sessions 1 and 5 of PavCA training significantly correlated with baseline CORT levels once a conditioned response had been acquired. Thus, while the relationship between baseline CORT and Pavlovian conditioned approach behavior is not apparent on a given day of training (early or late), the trajectory of CS-directed behavior (i.e., captured by Δ PavCA index) is linked to CORT. This notion is supported by previous findings that demonstrated phenotype-dependent responses in CORT immediately *following* the first Pavlovian conditioning session (Flagel et al., 2009); and those by Tomie et al. (2000) that showed a positive relationship between the acquisition of Pavlovian conditioned approach behavior (average behavior of sessions 1-10) and the CORT response immediately following a later training session. While the relationship between CORT and Pavlovian conditioned approach behavior is complex, the current data are indicative of an association between the propensity to attribute incentive salience to a food-cue, or the emergence of a sign-tracking conditioned response, and baseline CORT levels after that conditioned response is established.

One of the primary roles of CORT is to act across the body and brain to broadly mediate the stress response (Herman et al., 2016). Thus, we wanted to determine

whether differences in plasma CORT are present in goal-trackers vs. sign-trackers in contexts outside of Pavlovian conditioning and, explicitly, in response to paradigms reflective of negative valence. Experiment 1B showed no differences between phenotypes in CORT response to an open field test or physiological restraint. Further, goal-trackers and sign-trackers did not differ in their behavioral response to the open field test or elevated plus maze. These findings are consistent with those previously reported by Vanhille et al. (2015), who showed no pre-existing differences in behavior on the elevated plus maze test in rats that were later characterized as sign-trackers or goal-trackers; and those reported by Harb and Almeida (2014) who showed no differences in behavior on the open field test in mice characterized as sign-trackers or goal-trackers. In contrast to the present findings, however, Harb and Almeida (2014) did report a significant difference in peak CORT response following an acute stressor, with sign-tracker mice exhibiting a greater peak relative to goal-trackers or intermediate responders. These discrepant findings are likely due to differences in experimental procedures, including the species used and the nature and intensity of the stressor. In this regard, we note that the repeated testing implemented in the current study may have affected the CORT response in a manner that precluded observable differences (e.g. see Dallman et al., 2004). Indeed, it is possible that differences in the CORT profile in response to physiological restraint were not apparent because of a ceiling effect, as both baseline and peak CORT levels were high across all animals. Nonetheless, given that goal-trackers and sign-trackers behaved similarly on tests of negative valence, and showed no significant differences in CORT response to the open field test, we conclude

that individual differences in the negative valence system are not captured by the goal-tracker/sign-tracker animal model.

The RDoC includes fear conditioning and the associated freezing response within the domain of negative valence, specifically within the construct of acute threat or “fear”. Thus, it should be noted that goal-trackers and sign-trackers differ in their response to Pavlovian fear conditioning (Morrow et al., 2011; Morrow et al., 2015). Specifically, relative to goal-trackers, sign-trackers are more fearful of discrete cues that predict footshock (Morrow et al., 2011), and show exaggerated incubation of their fear response (Morrow et al., 2015). Importantly, however, goal-trackers exhibit greater contextual fear when placed back into a fear-conditioning context in the absence of discrete cues (Morrow et al., 2011). Thus, these differences seem to be specific to learning the value of the discrete cue, rather than differences in negative valence per se. While others have shown that CORT plays a critical role in fear conditioning, beyond the “stress” component (e.g., Marchand et al., 2007; Zorawski & Killcross, 2002), this has yet to be assessed within the context of the goal-tracker/sign-tracker animal model and will be the focus of future investigations.

The current findings and those of others (Harb & Almeida, 2014; Vanhille et al., 2015) demonstrate that goal-trackers and sign-trackers respond similarly to tests of negative valence. Yet, exposure to “stress” has been shown to alter the propensity to attribute incentive salience to reward cues (Fitzpatrick et al., 2019; Hynes et al., 2018; Lomanowska et al., 2011). Rats exposed to stress early in life exhibit greater sign-tracking behavior in adulthood (Hynes et al., 2018; Lomanowska et al., 2011). In contrast, adult rats exposed to a single prolonged stressor show an attenuation of sign-

tracking behavior (Fitzpatrick et al., 2019). Thus, the impact of “stress” on the propensity to sign-track appears to be dependent on the type of stressor and timing of exposure. In light of the current findings, we postulate that the neural processes underlying these reported stress-induced effects (Fitzpatrick et al., 2019; Hynes et al., 2018; Lomanowska et al., 2011) go beyond CORT and the HPA axis, and include components of the cortico-thalamic-striatal “motive” circuit, which is differentially engaged in sign-trackers vs. goal-trackers (Flagel, Cameron, et al., 2011; see also Kuhn et al., 2018).

One potential neural interface between “stress” and “motive” circuitry is the hippocampus (e.g., Maccari et al., 1991, for review, see Barr et al., 2017). Glucocorticoid receptors are densely expressed within the hippocampus (Reul & de Kloet, 1985) and CORT-GR interactions within this brain region play a critical role in the negative feedback system that acts to maintain homeostatic levels of CORT in the face of physiological or environmental challenges (Herman et al., 2012). Specifically, greater GR mRNA expression in the hippocampus has been associated with more rapid negative feedback, or return to baseline CORT levels (Liberzon et al., 1999; Meaney et al., 1996). In the current study, we did not observe phenotypic differences in circulating CORT levels either under baseline conditions or in response to environmental challenges (i.e., open field test or physiological restraint). Yet, we did find that, relative to goal-trackers and intermediate responders, sign-trackers have significantly greater expression of GR mRNA in the ventral hippocampus. The fact that these differences in GR mRNA expression are specific to the ventral hippocampus and not apparent in the dorsal hippocampus may explain why we did not observe differences in circulating levels of CORT. Indeed, while the dorsal hippocampus has been shown to play a role in

stress-induced negative feedback (Feldman & Weidenfeld, 1993, 1999), the ventral hippocampus has been reported to regulate tonic levels of CORT (Herman et al., 1992). Other findings suggest that the engagement of the ventral vs. dorsal hippocampus is dependent on the type of stressor (e.g., Dorey et al., 2012; Herman et al., 2005; Maggio & Segal, 2007). Thus, additional studies are warranted to further investigate the role of GR expression in the ventral hippocampus within the context of the stress response and negative feedback regulation. Nonetheless, we propose that the phenotypic differences reported here in GR mRNA expression in the ventral hippocampus are directly related to motivated behavior and reward learning, rather than stress regulation and negative valence. In support, lesions of the ventral, but not the dorsal, hippocampus decrease the propensity to sign-track (Fitzpatrick et al., 2016). While it is remains to be determined whether these lesion effects are dependent on GR function, it should be noted that systemic administration of a GR antagonist similarly attenuates the acquisition of sign-tracking behavior (Rice et al., 2018; Rice et al., 2019). Taken together, GR function, and presumably that within the ventral hippocampus, appears to play an important role in incentive motivational processes.

In conclusion, these findings establish that the neurobehavioral endophenotype associated with the propensity to sign-track does not include differences in response to tests with negative valence, including, EPM, OFT, and restraint. Further, we provide additional evidence that glucocorticoids, which have primarily been implicated in negative valence (but see Deroche et al., 1993; Piazza et al., 1993) are, in fact, involved in positive valence. Specifically, circulating levels of CORT appear to be linked to the propensity to attribute incentive salience to reward cues, or sign-tracking behavior. In

addition, expression of glucocorticoid receptors in the ventral hippocampus appear to be related to inherent cue-reward learning strategies rather than the stress response. These studies provide a critical foundation for future work to further examine the mechanism by which glucocorticoids interact with other neural systems known to play a role in incentive motivation (for further discussion, see Lopez & Flagel, 2020).

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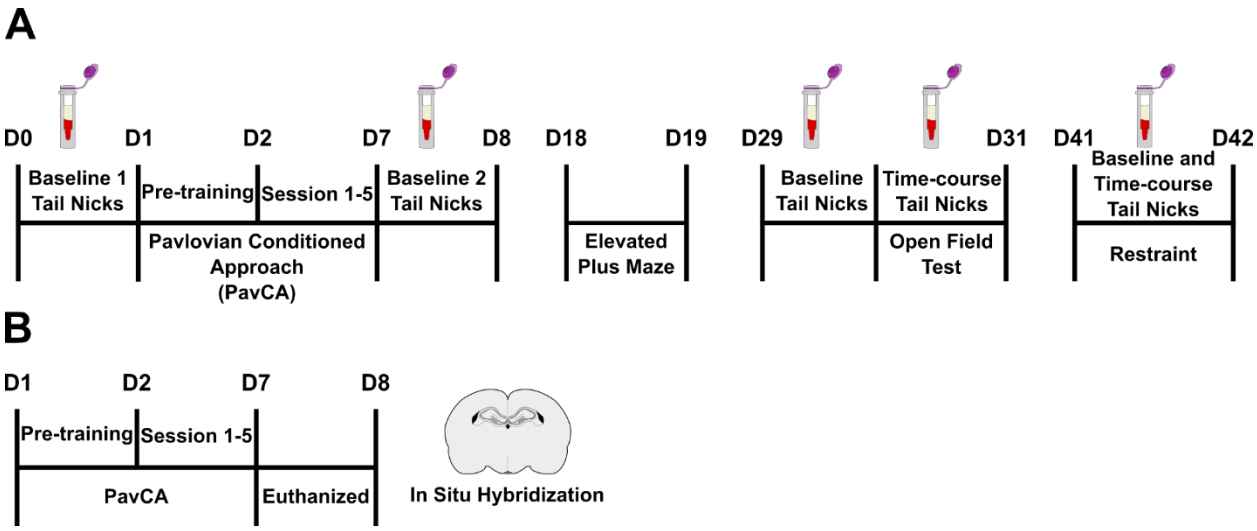

Figure 2.1. Experimental Timelines. A) "Baseline" tail nicks were performed for blood collection prior to Pavlovian conditioned approach (PavCA) training (Pre-PavCA), and after the rats had acquired a conditioned response (Post-PavCA). Rats were subsequently tested on an elevated plus maze (EPM) and the open field test (OFT), followed by physiological restraint, with a 10-day rest period prior to each. Corticosterone response to the OFT and acute restraint was captured with time-course blood sampling. **B**) A separate group of rats underwent 5 sessions of PavCA training and were subsequently euthanized to assess glucocorticoid receptor expression in the hippocampus using in situ hybridization.

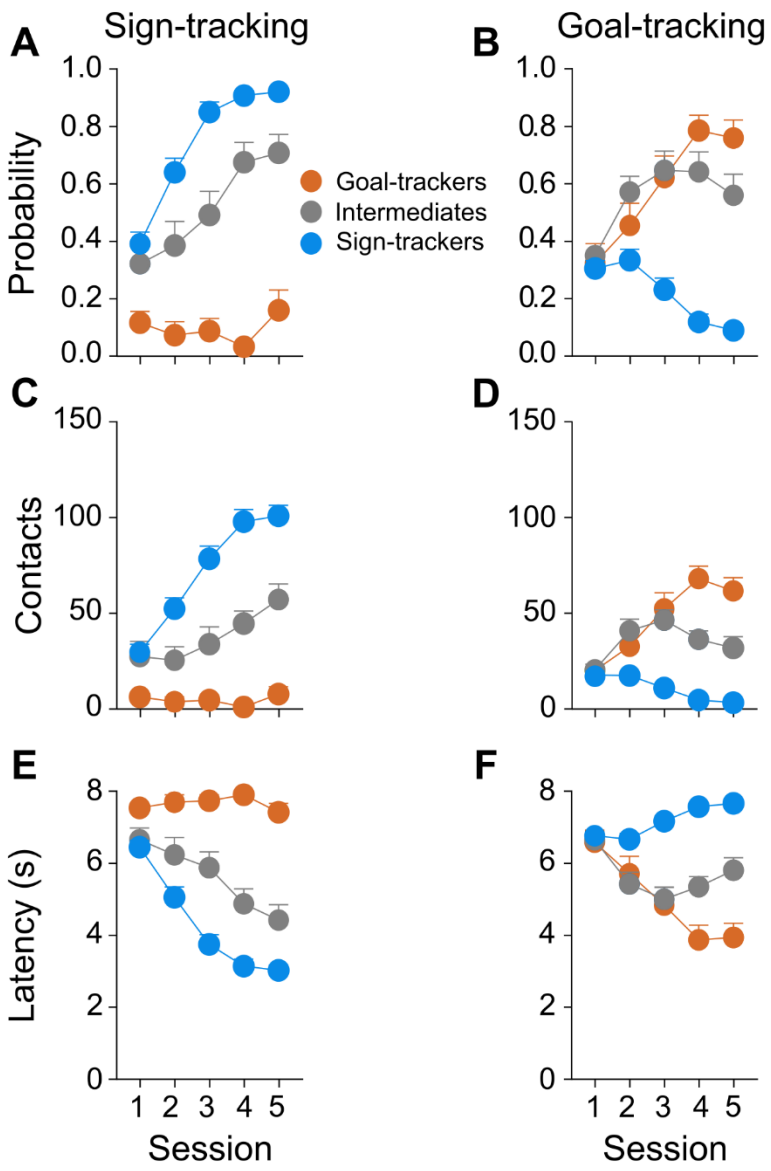

Figure 2.2. Acquisition of sign-tracking and goal-tracking behavior. Sign-tracking (i.e., lever-directed, left panels) and goal-tracking (i.e., food-cup directed, right panels) behavioral measures were assessed across 5 PavCA sessions. Mean + SEM for probability to: **A**) contact the lever or **B**) enter the food-cup, total number of contacts with **C**) the lever or **D**) the food-cup, and latency to **E**) contact the lever or **F**) enter the food-cup. Rats with a sign-tracking conditioned response were classified as STs (n= 32), those with a goal-tracking conditioned response as GTs (n= 11), and those that vacillated between the two conditioned responses as IRs (n= 17).

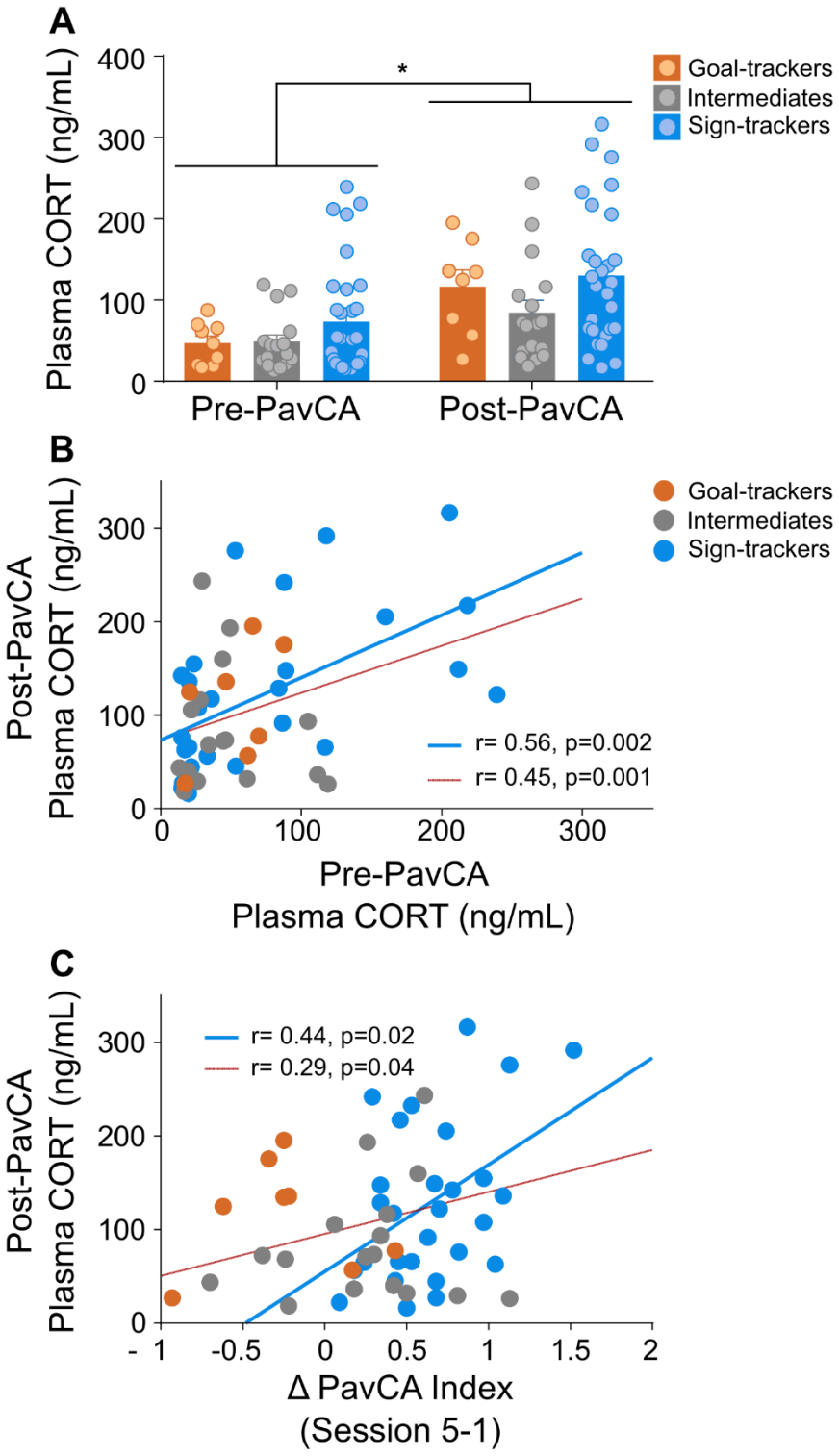

Figure 2.3. "Baseline" corticosterone levels before and after Pavlovian conditioned approach training. A) Mean + SEM for baseline plasma CORT levels prior to (Pre-PavCA) and following PavCA training experience (Post-PavCA). For all rats (n=60; GT n=11, IR=17, ST=32), the five sessions of PavCA training increased basal plasma CORT levels (*, p= 0.001). Bivariate scatterplots illustrating the relationship between **B**) Pre- and Post-PavCA baseline CORT levels, and **C**) the change in lever- vs. food-cup-directed behavior from sessions 1 to 5 (Δ PavCA Index) and Post-PavCA baseline CORT. The red line reflects the r value when all rats (n=59) are included, and the

blue line when only STs (n=27) are included. There is a significant positive correlation between Pre-PavCA and Post-PavCA plasma CORT levels, and between the ∆ PavCA Index and Post-PavCA plasma CORT levels. The latter is more apparent in STs.

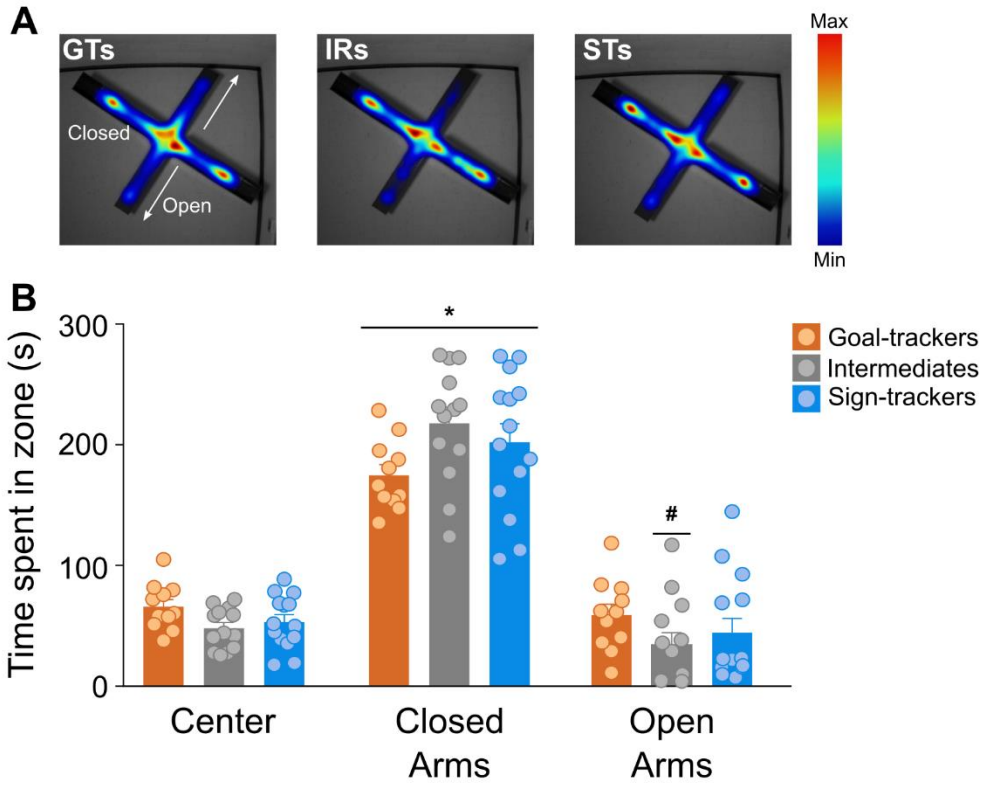

Figure 2.4. Elevated plus maze. A) Heat map representations for the average time spent in each zone during the 5-min EPM test for each phenotype. **B**) Mean + SEM for the time spent in each zone of the elevated plus maze for goal-trackers (n=11), intermediate responders (n=13), and sign-trackers (n=14). All rats spent significantly more time in the closed arms compared to the open arms and center of the maze (*, p<.001). There was not a significant difference between GTs and STs in the amount of time spent in either the center of the arena or the open or closed arms. IRs spent significantly less time in the open arms, relative to GTs (#, p<0.05).

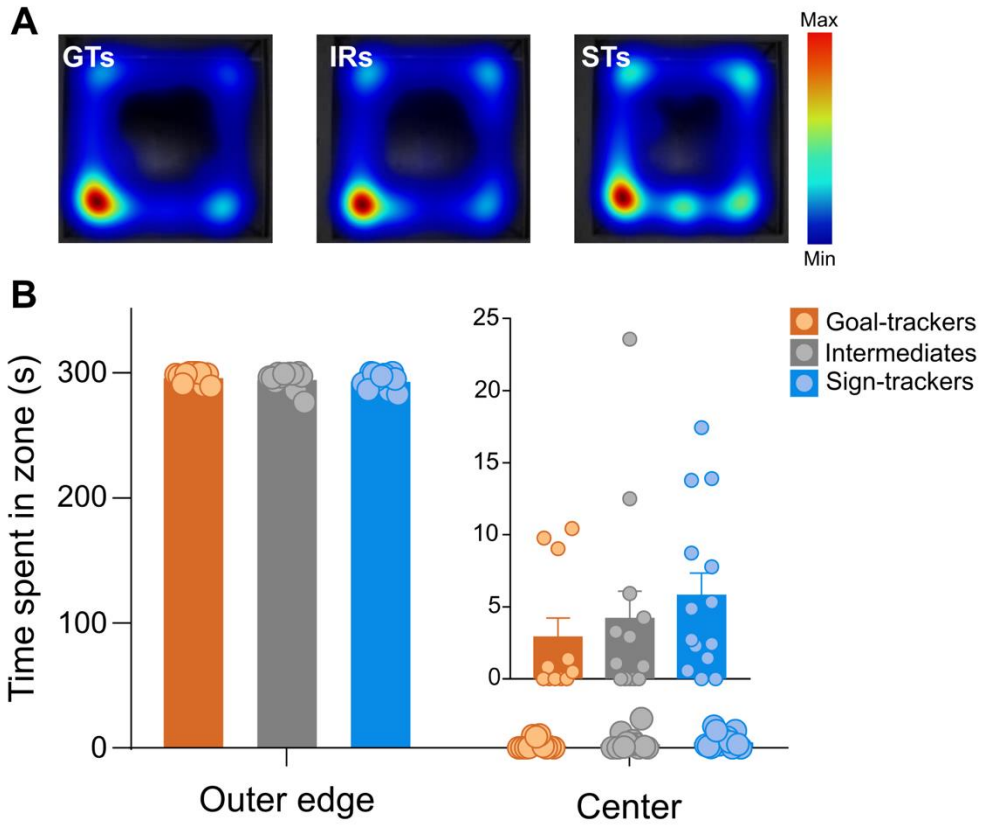

Figure 2.5. Open field test. A) Heat map representations for the average time spent in each zone (outer edge vs. center) during the 5-min OFT for each phenotype. **B**) Mean + SEM for time spent in the outer edge or center of the arena for goal-trackers (n=11), intermediate responders (n=13), and sign-trackers (n=14). All rats spent significantly more time on the outer edge of the arena compared to the center. Time spent in the center of the arena is shown as an inset on a different scale for illustration purposes. There was not a significant difference between phenotypes for the amount of time spent in the center of the arena.

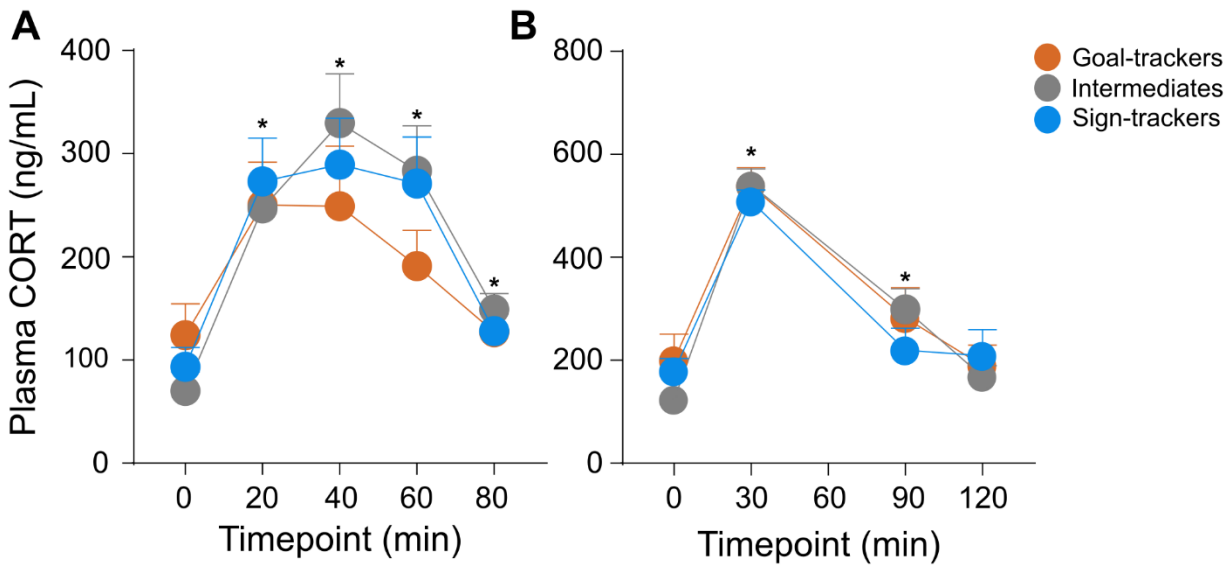

Figure 2.6. Corticosterone response to the open field test and acute physiological restraint. A) Mean + SEM for plasma CORT levels 0,20,40,60, and 80-mins post-onset of the OFT for goal-trackers (n=11), intermediate responders (n=13), and sign-trackers (n=14). There was a significant increase in CORT induced by the OFT at 20, 40, 60, and 80-min time-points (*, p<0.001), but no significant difference between phenotypes. **B**) Mean + SEM for plasma CORT levels 0,30,90, and120-mins post-onset of acute restraint for goal-trackers (n=11), intermediate responders (n=13) and sign-trackers (n=12). There was a significant increase in CORT induced by restraint at 30 and 90-min time-points (*, p<.001), but no significant difference between phenotypes.

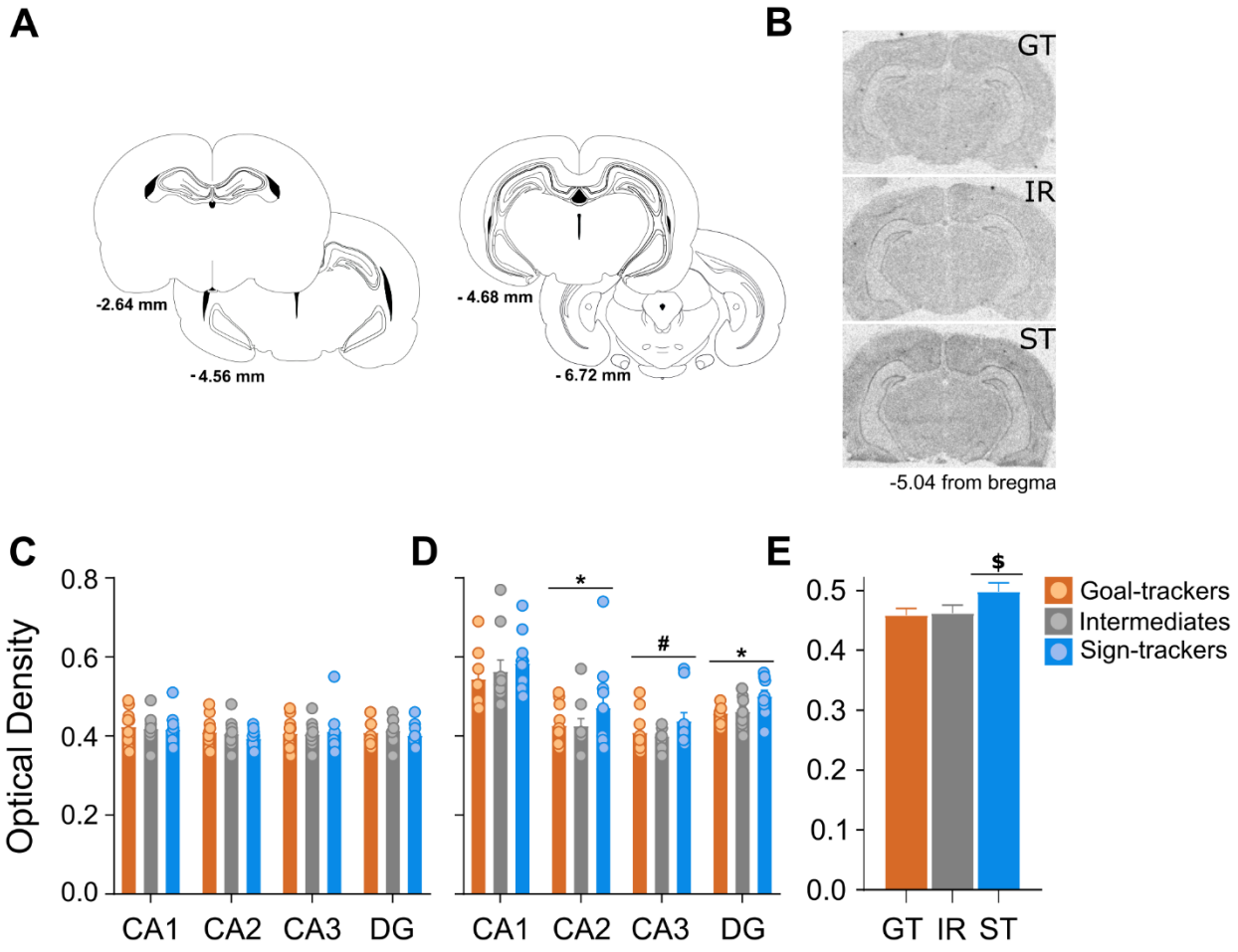

Figure 2.7. Glucocorticoid receptor mRNA expression in the dorsal and ventral hippocampus. A) Sagittal brain sections representing bregma coordinates used to quantify glucocorticoid receptor (GR) mRNA expression (*Adapted from* Paxinos and Watson, 2007). **B**) Representative in situ images for a GT, IR, and ST rat. **C-D**) Mean + SEM optical density for GR mRNA in subregions of the **C)** dorsal and **D)** ventral hippocampus for goal-trackers (n=10), intermediate responders (n=10) and sign-trackers (n=10). In the ventral hippocampus, GR mRNA varied between subregions (*, p<0.001 vs. CA1, #, p<0.001 vs. DG). **E**) Mean + SEM optical density for GR mRNA in the ventral hippocampus, subregions collapsed. Relative to goal-trackers and intermediate responders, sign-trackers show greater GR mRNA density ($, p<0.05).

PavCA Behavior

Experiment 1

Sign-tracking	Lever Contacts			Lever Contact Probability			Lever Contact Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Phenotype	2, 54.615	38.121	p<0.001	2, 57	56.803	p<0.001	2, 55.352	40.450	p<0.001
Effect of Session	4, 87.711	15.929	p<0.001	4, 57	23.654	p<0.001	4, 79.678	35.451	p<0.001
Phenotype*Session	8, 87.711	8.640	p<0.001	8, 57	9.027	p<0.001	8, 79.678	11.9.28	p<0.001

Goal-tracking	Food cup Entries			Food cup Entry Probability			Food cup Entry Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Phenotype	2, 63.287	52.264	p<0.001	2, 63.003	39.844	p<0.001	2, 60.931	45.807	p<0.001
Effect of Session	4, 96.698	14.610	p<0.001	4, 155.487	10.960	p<0.001	4, 81.979	14.380	p<0.001
Phenotype*Session	8, 96.698	16.953	p<0.001	8, 155.87	12.193	p<0.001	8, 81.979	17.313	p<0.001

Experiment 2

Sign-tracking	Lever Contacts			Lever Contact Probability			Lever Contact Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Phenotype	2, 25.738	73.590	p<0.001	2,27.026	111.836	p<0.001	2, 27.037	65.914	p<0.001
Effect of Session	4, 30.902	17.660	p<0.001	4, 25.527	37.054	p<0.001	4, 23.863	29.661	p<0.001
Phenotype*Session	8, 30.920	10.840	p<0.001	8, 25.527	17.592	p<0.001	8, 23.885	12.406	p<0.001

Goal-tracking	Food cup Entries			Food cup Entry Probability			Food cup Entry Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Phenotype	2, 31.080	39.402	p<0.001	2, 27.941	37.563	p<0.001	2, 26.445	41.540	p<0.001
Effect of Session	4, 39.514	5.3831	p= 0.002	4, 47.772	6.000	p= 0.001	4, 56.426	9.495	p<0.001
Phenotype*Session	8, 39.631	7.976	p<0.001	8, 47.731	10.586	p<0.001	8,56.453	12.399	p<0.001

Table 2.1. Results from Linear Mixed model analysis for sign-tracking (i.e., lever-directed) and goal-tracking (i.e., food-cup-directed) behaviors. Effect of Phenotype, Session, and Phenotype x Session interactions were analyzed for Experiment 1 (top) and Experiment 2 (bottom). Abbreviations: df_1, degrees of freedom numerator, df_2, degrees of freedom denominator.

Phenotype Comparisons

Experiment 1

	Sign-tracking Lever Contacts					**Goal-tracking** Food cup Entries				
	1	2	3	4	5	1	2	3	4	5
GT vs. IR	p= 0.090	p= 0.152	p= 0.083	p= 0.001*	p<0.001*	p= 1.000	p= 0.820	p= 1.000	p<0.001*	p<0.001*
GT vs. ST	p= 0.024*	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 1.000	p= 0.072	p<0.001*	p<0.001*	p<0.001*
ST vs. IR	p= 1.000	p= 0.007*	p<0.001*	p<0.001*	p<0.001*	p= 1.000	p<0.001*	p<0.001*	p<0.001*	p<0.001*

	Lever Contact Probability					Food cup Entry Probability				
	1	2	3	4	5	1	2	3	4	5
GT vs. IR	p= 0.074	p= 0.018*	p<0.001*	p<0.001*	p<0.001*	p= 1.000	p= 0.542	p= 1.000	p= 0.226	p= 0.051
GT vs. ST	p= 0.004*	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 1.000	p= 0.382	p<0.001*	p<0.001*	p<0.001*
ST vs. IR	p=0.930	p= 0.012*	p<0.001*	p<0.001*	p= 0.001*	p= 1.000	p= 0.002*	p<0.001*	p<0.001*	p<0.001*

	Lever Contact Latency					Food cup Entry Latency				
	1	2	3	4	5	1	2	3	4	5
GT vs. IR	p= 0.092	p= 0.057	p= 0.006*	p<0.001*	p<0.001*	p= 1.000	p= 1.00	p<0.001*	p<0.001*	p<0.001*
GT vs. ST	p= 0.011*	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 1.000	p= 0.081	p<0.001*	p<0.001*	p<0.001*
ST vs. IR	p= 1.000	p= 0.047*	p<0.001*	p<0.001*	p= 0.001*	p= 1.000	p= 0.003*	p<0.001*	p<0.001*	p<0.001*

Experiment 2

	Sign-tracking Lever Contacts					**Goal-tracking** Food cup Entries				
	1	2	3	4	5	1	2	3	4	5
GT vs. IR	p= 0.852	p= 0.005*	p= 0.016*	p= 0.001*	p<0.001*	p=0.959	p=1.000	p= 0.063	p<0.001*	p<0.001*
GT vs. ST	p= 0.001*	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 0.073	p= 0.052	p<0.001*	p<0.001*	p<0.001*
ST vs. IR	p= 0.010*	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 0.547	p=0.217	p= 0.001*	p<0.001*	p= 0.003*

	Lever Contact Probability					Food cup Entry Probability				
	1	2	3	4	5	1	2	3	4	5
GT vs. IR	p= 0.211	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 1.000	p=1.000	p= 0.820	p= 0.093	p= 0.005*
GT vs. ST	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 0.163	p= 0.138	p<0.001*	p<0.001*	p<0.001*
ST vs. IR	p=0.018*	p<0.001*	p= 0.004*	p= 0.003*	p= 0.003*	p= 0.331	p= 0.359	p<0.001*	p<0.001*	p<0.001*

	Lever Contact Latency					Food cup Entry Latency				
	1	2	3	4	5	1	2	3	4	5
GT vs. IR	p= 0.649	p= 0.005*	p<0.001*	p<0.001*	p<0.001*	p= 1.000	p= 1.00	p= 0.072	p<0.001*	p<0.001*
GT vs. ST	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p<0.001*	p= 0.285	p= 0.096	p<0.001*	p<0.001*	p<0.001*
ST vs. IR	p= 0.013*	p= 0.001*	p<0.001*	p<0.001*	p= 0.002*	p= 0.420	p= 0.408	p= 0.006*	p<0.001*	p<0.001*

Table 2.2. Bonferroni posthoc comparisons between phenotypes for each PavCA session. Sign-tracking (i.e., lever-directed) and goal-tracking (i.e. food-cup-directed) behaviors are included for Experiment 1 (top) and Experiment 2 (bottom). Abbreviations: GTs, goal-tackers, STs, sign-trackers, IR, intermediate responders. * $p<0.005$

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Chapter 3

The Effect of Systemic Corticosterone on the Acquisition and Expression of Goal- and Sign-tracking Behaviors in Male and Female Rats

Abstract

Through associative learning, environmental cues become predictors of relevant stimuli and facilitate adaptive behavior. However, when such cues are attributed with incentive value, they may gain excessive control and can trigger aberrant behavior. For example, when individuals with addiction encounter drug-cues, they often relapse, despite their awareness of the adverse consequences. Altered levels of glucocorticoids have been reported in individuals with cue-driven psychopathologies like addiction and post-traumatic stress disorder (PTSD), and it has been postulated that glucocorticoids may contribute to a common mechanism of susceptibility underlying these disorders. We hypothesize that glucocorticoids are specifically involved in incentive learning and that an inherent tendency for this form of learning may render one more susceptible to disorders like addiction and PTSD. In the current study we used the goal-tracker/ sign-tracker animal model, which allows us to parse predictive vs. incentive cue-learning, to test the hypothesis that systemic administration of glucocorticoids (in this case, corticosterone, CORT) would enhance the incentive value of a food cue in rats. We found that CORT increased the propensity to sign-track to a food-cue in male rats; while attenuating sign-tracking in female rats. When CORT treatment was terminated in

female rats, they showed a significant increase in sign-tracking behavior, suggesting that they were, perhaps, capable of attributing incentive value to the cue even under the influence of CORT. Further, CORT administration increased sign-tracking behaviors in female rats that had acquired a goal-tracking response. Additionally, the food-cue attained greater reinforcing properties in male rats, having learned cue-reward associations under high CORT levels, as they more readily worked for its presentation. It appears, therefore, that altered levels of CORT influence incentive cue-learning in a sex- and phenotype-dependent manner.

Introduction

The way individuals respond to cues in the environment may be indicative of vulnerability to psychopathology. For some, the learned association between a cue and a stimulus (e.g., a valuable resource) can influence behavior in adaptive ways. For others, however, such cues can attain incentive properties and may trigger aberrant behaviors (Robinson & Berridge, 1993). The latter form of learning – incentive learning – can be relevant to psychopathology. For example, individuals with addiction often relapse upon contact with cues (e.g. paraphernalia, people) previously associated with the drug-taking experience (Childress et al., 1993). Similarly, those with post-traumatic stress disorder (PTSD) become hyperaroused upon exposure to trauma-associated stimuli (Vermetten & Bremner, 2003). Interestingly, altered glucocorticoid (GC) levels have been reported for individuals with addiction and PTSD (for review, see, Hadad et al., 2020). For those diagnosed with addiction, GC levels rise in response to drug-associated cues (Sinha et al., 2003) and are elevated during the initial drug-abstinence period (Contoreggi et al., 2003). Following a traumatic experience, low GC levels are

thought to be predictive of the development of PTSD in the future (Delahanty et al., 2000). Low GC levels have also been reported in those diagnosed with PTSD (Pan et al., 2020). Not surprisingly, alterations in GC-function have been discussed in the context of addiction and PTSD comorbidity, including relevance to motivational salience attribution (for review, see Hadad et al., 2020; Maria-Rios & Morrow, 2020). However, our understanding of the role of glucocorticoids in incentive cue-reward learning is limited.

The goal-tracker/ sign-tracker animal model can be exploited to investigate the neurobiology driving individual differences in predictive vs. incentive cue-reward learning (Robinson & Flagel, 2009). In rodents, the sign-tracking phenotype (i.e., incentive) encompasses traits relevant to psychopathologies like addiction and PTSD. For example, sign-trackers attribute incentive value to drug-paired cues (Uslaner et al., 2006) and show an increased and exaggerated fear response to aversive cues (Morrow et al., 2011; Morrow et al., 2015). To date, it has been demonstrated that plasma corticosterone (CORT) levels, the main glucocorticoid in rodents, rise to a greater extent in male sign-tracker rats, relative to goal-trackers (Flagel et al., 2009). While GCs are often associated with negative valence systems (RDoC), data from Chapter 2, suggest that this increase in plasma CORT pertains to differences in cue-reward learning.

Pharmacological manipulation of CORT and its Type II receptors (i.e., GRs) has long been known to influence Pavlovian associations in the context of cognition and learning and memory (Cordero & Sandi, 1998; Mormede & Dantzer, 1977). Most of this work has focused on the association between context and aversive stimuli (e.g., Cordero et al., 2002). Few studies, however, have identified a role for CORT in discrete-

cue and appetitive stimulus associations (e.g.,Zorawski & Killcross, 2002). In relation to goal- and sign-tracking, a study using male Japanese quail demonstrated a role of GRs in the propensity to sign-track to a key-light cue (Rice et al., 2018). Quail that voluntarily-administered (i.e., orally) a GR-antagonist prior to Pavlovian conditioning sessions showed an attenuation of sign-tracking behavior, relative to their placebo counterparts (Rice et al., 2018), and this effect is dose-dependent when administered systemically (Rice et al., 2019).

The goal of the current studies was to expand our understanding of altered CORT-GR function in cue-reward learning. Specifically, we hypothesized that, in contrast to a GR antagonist, systemic administration of CORT, the endogenous GR agonist, would enhance sign-tracking behaviors. To assess the effect of elevated CORT on the *acquisition* of a conditioned response to a reward-cue, a cohort of male rats (Experiment 1) and two cohorts of female and male rats trained in conjunction (Experiment 2) received a systemic injection of either vehicle (VEH) or CORT prior to each Pavlovian conditioned approach (PavCA) training session. To assess the effect of elevated CORT on the *expression* of a learned conditioned response, treatment was reversed in a subset of rats from Experiment 2. Thus, rats that received CORT during acquisition now received VEH during two additional PavCA sessions, and those who previously received VEH received CORT prior to these two additional sessions. The remaining rats from Experiment 1 and 2 underwent a conditioned reinforcement test to assess the effect of altered CORT history on the reinforcing properties, or incentive value, of the cue.

Materials and Methods

Animals

A total of sixty female and eighty-four male Sprague-Dawley rats, weighing between 225-275 g, arrived from Charles River (colony R04 and R08, Raleigh, NC, USA) and from Taconic Biosciences (colony Bu016, Cambridge City, IN, USA). Experiment 1 consisted of one round with just males (n= 24), and Experiment 2 consisted of two rounds with both males (n=60, 30/round) and females (n=60, 30/round). Upon arrival, rats were paired-housed with the same sex in standard acrylic cages (46 x 24 x 22 cm) in a temperature-controlled (22 ± 2°C) housing room with a 12 hr light: dark cycle (lights on at 06:00 or 07:00 depending on daylight savings time) for the duration of the study. All rats had ab libitum access to food and water. Rats were left undisturbed and allowed to acclimate to the housing conditions for seven days after arrival. Prior to experimental manipulations, rats were briefly handled for two consecutive days and received twenty-five 45 mg banana-flavored grain pellets (Bio-Serv, Flemington, NJ, USA) inside the homecage. This, allowed rats to habituate to the food reward used during Pavlovian conditioned approach training. All experimental procedures took place during the light phase of the cycle (between 09:00 to 16:00). Guidelines put forth in *The Guide for the Care of and Use of Laboratory Animals: Eighth Edition* (2011, National Academy of Sciences) were followed and all experimental procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

Monitoring the estrous cycle

Female rats were monitored daily (~15:00 h) for their stage of estrous cycle by vaginal lavages. Lavages were performed for at least two days before behavioral testing

and continued for the duration of the study. Procedures were similar to those described by Becker et al. (2005). The tip of a glass eyedropper filled with sterile 0.9% saline was inserted into the vaginal opening and gentle pulsations, via slight bulb pressure, were used to extract cells. Each sample was placed into a well (of a 24-well plate), and the dropper was rinsed thoroughly with distilled water three times between each rat. Vaginal epithelial cell cytology of each sample was observed under an inverted light microscope (20X). Estrous cycle stage characterization was determined based on the following criteria and similar to (Alonso-Caraballo & Ferrario, 2019; Hubscher et al., 2005): *(M) Metestrus*- a mix of leukocytes, cornified cells, and round cells, *(D*) *Diestrus*- mainly leukocytes and some nucleated epithelial cells*, (P) Proestrus* – mainly nucleated epithelial cells that form sheets, *(E) Estrus*- large cornified cells lacking nuclei. Weight was also tracked daily; as rats were expected to be at their heaviest during *Diestrus* and their lightest during *Estrus*. Males were weighed daily to account for any "extra" handling. Given the longitudinal design of the study, it was difficult to assess and identify effects of the estrous cycle on the behavioral outcome measures. Thus, these data are not shown here.

Behavioral testing

Pavlovian conditioned approach (PavCA)

As described in Chapter 2, PavCA took place in standard testing chambers (MED Associates, St. Albans, VT, USA; 20.5 × 24.1 cm floor area, 29.2 cm high) located inside sound-attenuating boxes. A ventilation fan masked background noise. Each chamber was outfitted with a food-cup centered on one of the 20.5 cm walls. Food-cup entries were detected by breaks of an infra-red beam located inside. A retractable lever, that

was illuminated upon its presentation, was located either to the right or the left of the food-cup. Lever contacts were detected when a force of at least 10 g was used to deflect it. On the opposite 20.5 cm wall, a white house light was located at the top of the chamber. All behavioral testing was performed under red lighting.

Rats underwent at least one pre-training session. If any rats did not consume all of the food-pellets by the end of the pre-training session an additional session was conducted for that experimental round. No more than two pre-training sessions were conducted. Prior to each pre-training session, the food-cup was baited with three grain-pellets to direct the rat's attention to the location of reward delivery. Once placed in the chamber the house light turned on after 5 min to signal the beginning of the session and remained on through the entirety of the session. The session consisted of 25 trials during which the lever remained retracted, and pellets were delivered randomly into the food-cup; one pellet per trial on a variable interval 30 s schedule (range 0-60 s). The total session length was approximately 12.5 min.

Twenty-four hours after the last pre-training session, rats underwent a total of five (Experiment 1 (males (n=24)) and Experiment 2 (females (n=60), males (n=60)) or seven (a subset of rats from Experiment 2 (females (n=29), males (n=30)) consecutive PavCA training sessions (see experimental timeline Figure 3.1). Each session consisted of 25 trials on a variable interval 90 s schedule (VI 90, range 30-150 s) during which the illuminated lever (conditioned stimulus, CS) was presented for a total of 8 s, and immediately upon its retraction, a food pellet (unconditioned stimulus, US) was delivered into the adjacent food-cup. Each session lasted approximately 40 min.

The following data were recorded during each PavCA session: (1) number of lever contacts, (2) latency to contact the lever for the first time, (3) probability to contact the lever, (4) number of food-cup entries during presentation of the lever, (5) latency to first enter the food-cup during presentation of the lever, (6) probability of entering the food-cup during presentation of the lever, and (7) number of food-cup entries during the inter-trial interval. These values were then used to calculate three measures of approach behavior that comprise the PavCA index: (1) response bias [(total lever presses – total food-cup entries) ÷ (total lever presses + total food-cup entries)], (2) probability difference [probability to approach the lever – the probability to enter the food-cup], (3) latency difference [± (latency to approach the lever – latency to enter the food-cup) ÷ 8]. The PavCA index score was then calculated using this formula (Meyer et al., 2012): [(response bias + probability difference + latency difference) ÷ 3]. Scores ranged from +1 to -1. Given the focus of this study was to assess the effect of treatment (see Pharmacological treatment section below) on the acquisition of a conditioned response, rats were not characterized as goal-trackers (GTs), intermediate responders (IRs), and sign-trackers (STs) for the majority of analyses and graphical representations. Rather, a more positive score indicated a preference for sign-tracking behavior and a negative score for goal-tracking.

Conditioned reinforcement test

Following the completion of PavCA training (session 1-5), a subset of rats (Experiment 1 (males (n=22)) and Experiment 2 (females (n=28), males (n=28)), those that did not move on to a 6th and 7th PavCA session (see experimental timeline, Figure 3.1), underwent a conditioned reinforcement test (CRT) to assess the effect of prior

CORT administration (i.e., during PaVCA) on the conditioned reinforcing properties of the lever-CS. Testing chambers were rearranged so that the food-cup was removed, and in its place, the lever was centered on the wall. Two nose ports were placed to the right and left of the lever. The nose port placed opposite to the lever's original position was designated the "active" port, for which nose pokes resulted in a 2-sec presentation of the illuminated lever. The other nose port was designated "inactive" and responses into it were without consequence. The house light turned on 1 min after rats were placed into the chamber and remained on for the duration of the session. The number of pokes into the active and inactive nose ports, as well as, the number of lever contacts were recorded during the session. A composite incentive value index (see Hughson et al., 2019) was calculated using the following formula: [(active pokes + lever contacts) – (inactive pokes)]. The session lasted approximately 40 min.

Pharmacological treatment

Acquisition: Experiment 1 and 2

Immediately following the last pre-training, all rats were transported into a designated room under red light and received an intraperitoneal (i.p.) injection of vehicle (VEH, 5% dimethylsulfoxide (DMSO) in sesame oil) in order to familiarize them with the injection procedure. Rats were subsequently assigned to either a VEH or corticosterone (CORT) treatment group, counterbalanced by vendor. To assess the effect of systemic CORT on the acquisition of a conditioned response, rats received an i.p. injection of VEH or 3 mg/kg CORT prior to PavCA sessions 1-5. They were left inside the injection room undisturbed for 30 min before being taken into the behavioral testing room for PavCA training.

Reversed treatment: Experiment 2

For a subset of rats (see experimental timeline, Figure 3.1), prior to sessions 6 and 7, treatment was reversed; those that had previously received VEH were injected with CORT, and those that received CORT were injected with VEH.

Statistical analysis

Behavioral outcome measures were analyzed using the Statistical Package for the Social Sciences (SPSS) program version 26.0 (IBM, Armok, NY, USA). Linear mixed-effects models were performed for PavCA behavior, using the best fit covariance structure with the lowest Akaike's information criterion for each set of data. Univariate analysis of variance was performed for conditioned reinforcement test measures. Statistical significance was set at $p<0.05$, and Bonferroni post hoc comparisons were conducted when significant interactions were detected. For a single analysis, vehicle group rats were characterized as GTs, IRs, and STs, using the averaged PavCA index from sessions 4 and 5 (Meyer et al., 2012). Index cutoffs were identical to Chapter 2: ≤ -0.5 for a GT, ≥ 0.5 for a ST, and in between -0.5 and 0.5 for an IR. Three rats were excluded from analysis due to failure to consume the reward for multiple sessions. All figures were made using GraphPad Prism 7.

Results

Acquisition

Pavlovian conditioned approach (PavCA) behavior

Experiment 1: Males only

To assess the effects of corticosterone (CORT, n=12), relative to vehicle (VEH, n=11), on the acquisition of a conditioned response, lever and food-cup directed behaviors were captured across five consecutive PavCA training sessions and compared between treatment groups. PavCA behavior measures included, total number of contacts, probability, and latency to approach either the lever or food-cup; and, main Effects of Treatment, Session, and Treatment x Session interactions are reported in Table 3.1 (top). Pairwise comparisons between treatments are reported in Table 3.2 (top). There was a significant Effect of treatment and session for all lever-directed behaviors, with CORT treated rats showing a greater number of contacts, higher probability, and lower latency to approach the lever, relative to those treated with vehicle. In contrast, food-cup directed behaviors were not significantly different between treatment groups. Overall, rats receiving CORT prior to each training session, showed a significantly higher propensity to sign-track, as measuerd by the PavCA index, relative to those receiving VEH ([Effect of Treatment: $F_{1,25.080}$=5.639, *p*=0.026], Figure 3.2A). While there was a significant effect of session [Effect of Session:$F_{4,79.020}$=2.502, *p*=0.049], the effect of treatment was not dependent on session [Treatment x Session interaction: $F_{4,79.020}$=1.406, *p*=0.240]. Together, these data show that systemic administration of CORT enhances the acquisition of sign-tracking behavior in male rats.

Experiement 2: Females vs. Males

To assess the effect of CORT on the acquisition of a conditioned response in female vs. male rats, lever- and food-cup-directed behaviors were captured across five consecutive PavCA sessions and compared between Treatment and Sex (Female: VEH (n=31), CORT (n=28); Male: VEH (n=31), CORT (n=29)). Reported on Table 3.1

(bottom), there was a significant Effect of sex for all PavCA outcome measures. Thus, PavCA Index was analyzed separately for female and male rats to determine the effect of CORT on the propensity to either sign-track or goal-track for each sex. Overall, systemic CORT decreased the propensity to sign-track for female rats [Effect of Treatment: $F_{1,57}$=3.930, *p*=0.052], independent of session [Effect of Session:$F_{4,57}$=15.885, *p*<0.001; Treatment x Session interaction: $F_{4,57}$=0.360, *p*=0.836] (Figure 3.2B). Interestingly, the PavCA index increased across sessions for male rats [Effect of Session:$F_{4,58}$=5.26, *p*=0.001]; but when male rats were trained in conjunction with females, CORT appeared to have no effect on the propensity to either goal- or sign-track [Effect of Treatment: $F_{1,58}$=0.475, *p*=0.494; Treatment x Session interaction: $F_{4,58}$=1.451, *p*=0.229]. Thus, we were unable to replicate the effects found in Experiment 1 with male rats.

Reversed treatment

Pavlovian conditioned approach (PavCA) behavior

Experiment 2: Females and Males

For a subset of male (CORT (n=15)) and female rats (CORT (n=14)), the effect of removing CORT treatment, and adminstering VEH in its place was assesed across two additional PavCA training sessions (6 and 7, Figure 3.3A,D). Additionally, a subset of rats (male:VEH (n=15), female:VEH (n=15)) that received VEH during acquisition were subsequently administered CORT on sessions 6 and 7, after the conditioned response had been acquired (Figure 3.3B,E). To assess the effects of these “reversed treatments” sessions 5, 6 and 7 were compared using linear mixed model analysis. For

female rats, removing CORT treatment resulted in increased sign-tracking behavior [Effect of Session:$F_{2,21.141}$=3.803, p=0.039]. Specifically, sign-tracking behavior is greater on Session 6 (p=0.05), the first day receiving VEH, relative to Session 5, the last day receiving CORT (Figure3.3A). Session 7, however, is not significantly different from Session 5 (p=0.059) or 6 (p=1.00). Adminstering CORT, instead of VEH, did not significantly affect female rats [Effect of Session: $F_{2,13.827}$=2.842, p=0.093] (Figure 3.3B). However, if split by phenotype based on the average PavCA Index of sessions 4 and 5, there is a significant Effect of Session [$F_{2,11.887}$ =33.50, p<0.001], Phenotype [Effect of Phenotype:$F_{2,12.084}$=45.290, p<0.001], and a Session x Phenotype interaction [$F_{4,11.874}$=25.054, p<0.001] (Figure 3.3C). Female GTs exhibited greater sign-tracking behavior in response to CORT such that the PavCA Index on Session 7 was significantly greater than that on Session 5 (p=0.007) and 6 (p<0.001). Thus, in females, CORT appears to suppress the acquisition of sign-tracking behavior, but after the conditioned response is learned, CORT increases the tendency to sign-track selectively in GT rats.

For males trained in conjunction with females, removing CORT treatment [Effect of Session: $F_{2,16.5047}$=1.161, p=0.337] (Figure 3.3 D) or initiating CORT treatment [Effect of Session:$F_{2,28}$=0.136, p=0.873] (Figure 3.3E) did not have a significant effect on PavCA Index. Further, asssessing the effect of CORT as a function of Phenotype did not capture a significant effect in males based on their learned conditioned response [Effect of Session:$F_{2,16.248}$ =0.102, p=0.90; Effect of Phenotype:$F_{2,12.}$=39.589, p<0.001; Session x Phenotype interaction: $F_{4,16.248}$=1.722, p=0.194] (Figure 3.3F). Thus, CORT did not appear to affect the expression of the conditioned response in male rats. Further

investigation of the influence of training males and females in parallel vs. separately is warranted.

Conditioned reinforcement test (CRT)

Experiment 1: Males only

To assess the incentive value of the lever-CS, a conditioned reinforcement test (CRT) was conducted following PavCA training, and no additional treatment was administered prior to the test. Data are reported in Table 3.3 (top). Overall, male rats that received CORT prior to PavCA training exhibited greater nosepokes compared to those that received VEH, but this effect was not specific to either port. Once the lever was presented, however, male rats that previously received CORT engaged with the lever to a greater extent than their VEH-treated counterparts. The composite incentive value index reflects these differences, as there is a significant Effect of treatment [$F_{1,20}$=4.639, p=0.044], with CORT-treated rats exhibiting a greater incentive value index (Figure 3.4A). It is important to note that this effect is primarily carried by greater lever contacts, given that they are not discriminating between nose ports.

Experiment 2: Females and Males

CRT behavior of female (VEH (n=14), CORT (n=14)) and male (VEH (n=14), CORT (n=14)) rats, that did not undergo treatment reversal (see experiemntal timeline, Figure3.1) is reported in Table 3.3 (bottom). Females, had greater nosepokes into the active port, but this was not depedent on treatment history, nor was the incentive value index [Effect of Treatment: $F_{1,26}$=0.505, p=0.484] (Figure 3.4B). Male rats that previously received CORT exhibited a greater number of nosepokes into the active port relative to

VEH-treated rats, but there was not a significant effect on lever contacts nor the incentive value index [Effect of Treatment: $F_{1,26}$=1.472, *p*=0.236] (Figure3.4C). In summary, while CORT had no effect on the acquisition or expression of PavCA behavior in males tested concurrently with females, it appears that prior CORT administration alters the conditioned reinforcing properties of the lever-CS for these male rats.

Discussion

These studies revealed that systemic administration of corticosterone (CORT) influences the propensity to attribute incentive value to reward-cues, and that it does so in a sex- and phenotype-dependent manner. The main findings include: 1) opposing effects of CORT on the acquisition of a conditioned response in male and female rats, with an enhancement of sign-tracking behavior in males (Experiment 1) and an attenuation in females (Experiment 2); 2) a release of the "suppressing" effect of CORT on sign-tracking in females, once treatment is terminated, 3) a CORT-elicited increase in the expression of sign-tracking behaviors for female rats that had acquired a goal-tracking response; and 4) an enhancement of the reinforcing properties of the lever-cue for male rats that learned the cue-reward association under elevated levels of CORT.

The hypothesis that systemic CORT administration would increase the propensity to attribute incentive value to reward-cues was derived largely from observations in studies with quail. Voluntary (Rice et al., 2018) and experimenter-administered (Rice et al., 2019) glucocorticoid receptor (GR) antagonist (PT150) attenuated key-light-cue approach behaviors (i.e., sign-tracking) in male Japanese quail. Thus, we expected the opposite following administration of CORT, a GR agonist.

The current studies are the first to investigate the role of CORT, using a pharmacological approach, in incentive vs. predictive learning.The dose of 3 mg/kg i.p. was selected, as previous studies (Dietz et al., 2007) have demonstrated that similar doses (2.5 mg/kg i.p.) increase plasma CORT levels (~ 300 ng/mL) equivalent to those reported to have reinforcing properties (Piazza et al., 1993) and increase dopamine levels within the nucleus accumbens (Piazza et al., 1996). Importantly, this prior research involved only male rats. Yet, we know that females, relative to males, have greater plasma CORT levels that rise more rapidly in response to stressors (Kant et al., 1983) and the response to exogenous CORT (e.g., via implanted pellets), is believed to be influenced by gonadal hormones (Young, 1996). Further, CORT administration in the context of fear-conditioning has opposite effects on the conditioned behavior of male vs. female mice (Lesuis et al., 2018). Thus, it is not unreasonable to expect sex differences underlying the role of glucocorticoids in appetitive behaviors and, specifically, in incentive learning processes.

In support of our hypothesis, the administration of CORT, before each of five consecutive Pavlovian conditioning sessions, increased the propensity to sign-track in male rats. The opposite was observed in females, with an attenuation of sign-tracking behavior. In general, we often detect a skewness towards sign-tracking in female rats, which is not apparent in males (see Hughson et al., 2019). Thus, the distribution of phenotypes may have contributed to these opposing effects. Not knowing the inherent tendencies of a given animal is a caveat of "acquisition" studies.

One of the more intriguing findings in these studies was the fact that we were unable to replicate the robust effects we observed with CORT administration in male

rats when males were trained and tested in conjunction with female rats. Given the only experimental difference between Experiment 1 males and Experiment 2 males was the presence of females, this perhaps influenced the inconsistent results in male rats. While males and females never directly interacted, their continuous presence within the housing and testing room might have been sufficient to have an effect. Specifically, males and females were in a homecage with the same sex, but cage placement in the colony room was counterbalanced (e.g., male, female, male). In the testing room, males and females never shared a testing chamber, but placement into each chamber was counterbalanced by sex. The same experimenter handled both males and females and neither gloves nor gowns were switched in between rats. Additionally, the same scale was used to weigh males and females daily and it was not cleaned in between rats. Previous studies have demonstrated that the mere presence of female rats can increase plasma CORT levels and adrenal weight in males (Lemaire et al., 1997), while others have found this effect to be opposite when males are under high-stress, decreasing CORT and adrenal weights (Taylor et al., 1987). Therefore, the presence of females in Experiment 2 might have influenced male behavior – or vice versa – and the impact could have been further confounded by treatment with CORT. Interestingly, regardless of being trained separately or in conjunction with females, male rats with previously elevated CORT history appear to "work" more readily for the presentation of the lever-CS and interact with the lever to a greater extent. That is, the lever-CS attains graeter reinforcing properties for male rats with history of altered CORT. Notably, we lack understanding of the effect of CORT on females without the presence of males, and this warrants further investigation.

While understanding the effect of CORT on the acquisition of a conditioned response, without knowing what their inherent tendencies are is a challenge, the "reverse treatment" experiment suggests that CORT may be "suppressing" sign-tracking behavior in females during acquisition and, once removed, their preferred sign-tracking response is recovered. This suggests they learned the cue-reward association and attributed incentive value, but it was not apparent until CORT treatment was removed. Further, the expression of sign-tracking behaviors in female rats, those that learned a conditioned response under vehicle, was not affected by two consecutive days of CORT treatment. Whereas, administering CORT for the first time in female goal-trackers, however, does increase lever-directed behaviors. However, male rats trained in conjunction with females, were not responsive to the removal or adminstration of CORT once a conditioned response was acquired. Thus, systemic CORT is not only influencing behavior distinctly between sexes but appears to do so as a function of Phenotype and time period of administration - acquistion vs. expression.

In conclusion, systemic administration of CORT can influence the propensity to attribute incentive value to reward-cues. The same dose differentially influences cue-induced behaviors in male vs. female rats, and female goal-trackers appear to be the most susceptible to changes induced by CORT once a conditioned response is established. The situational (e.g., presence of females) and sex-dependent effects of CORT captured by these studies embrace the complexity of glucocorticoids, which may be relevant to the aberrant behaviors manifested in psychopathologies like addiction and PTSD. Future studies will focus on local interactions of this hormone within brain structures implicated in predictive vs. incentive learning of both male and female rats.

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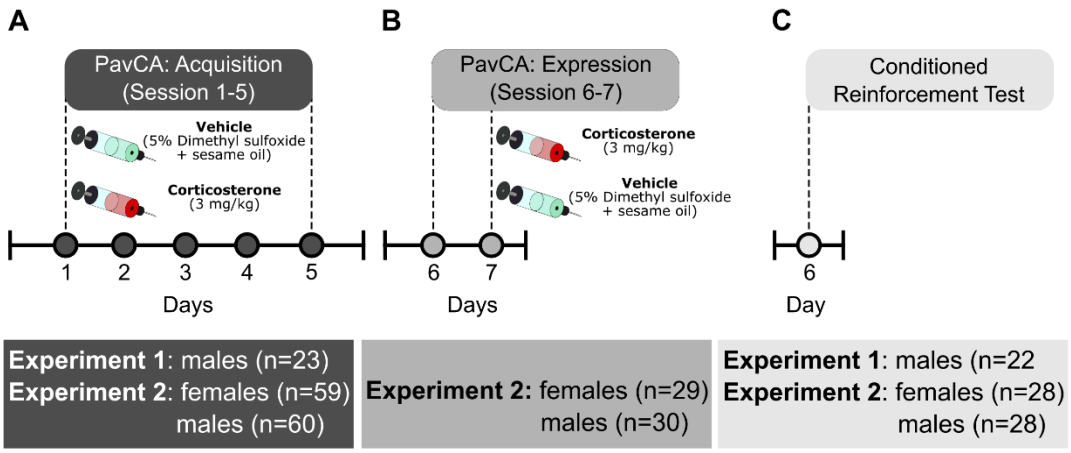

Figure 3.1 Experimental timeline. A) Experiment 1 (n=23) and 2 (n=119) rats were systemically administered with vehicle (VEH), 5% dimethyl sulfoxide (DMSO) diluted in sesame oil, or 3 mg/kg corticosterone (CORT) thirty minutes prior to each of five consecutive Pavlovian conditioned approach (PavCA) training sessions. On the sixth PaVCA session, rats either **B**) had their treatment reversed and underwent two additional PavCA sessions, session 6 and 7 (Experiment 2 (n=59); or **C**) underwent a treatment-free single conditioned test (CRT) (Experiment 1 (n=22) and 2 (n=56).

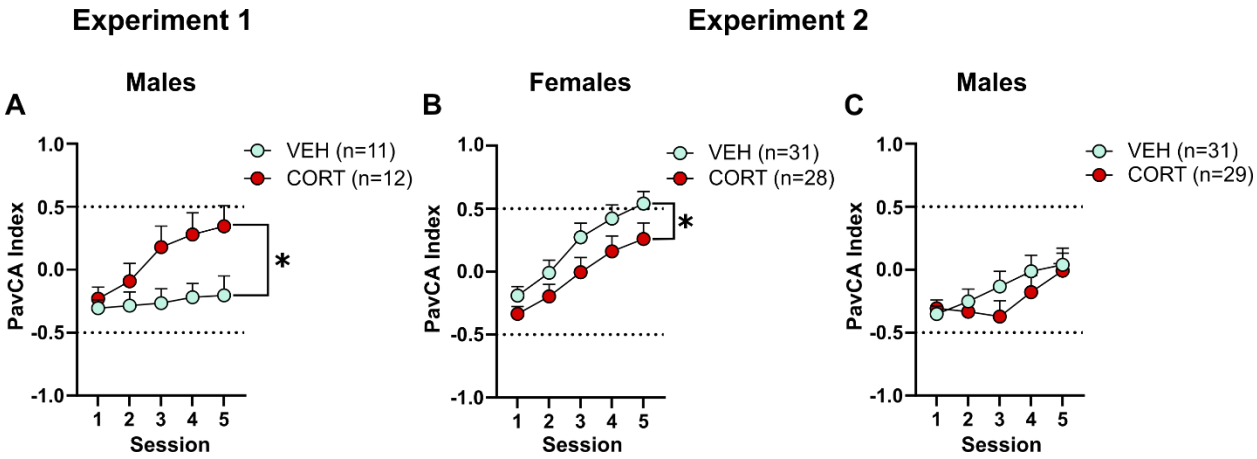

Figure 3.2 Effect of systemic corticosterone on the acquisition of sign- and goal-tracking behaviors in male (Experiment 1) and female vs. male (Experiment 2) rats. Mean + SEM for PavCA Index of **A**) Experiment 1 males, **B**) Experiment 2 females, and **C**) males trained in conjunction with females. CORT significantly increases the propensity to sign-track for **A**) males (p=0.026), while it decreases it for **B**) females (p=0.052). When **C**) males are trained in conjunction with females, however, CORT has no effect on PavCA Index(p=0.494).

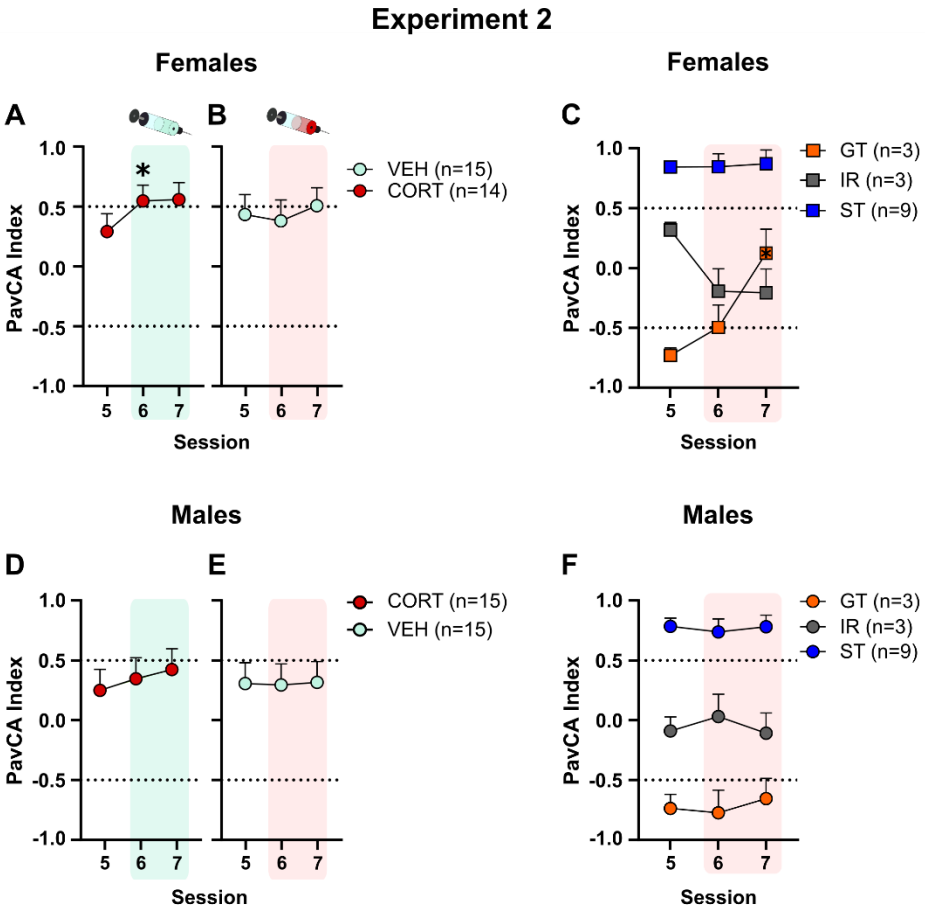

Figure 3.3 Effect of systemic corticosterone on the expression of sign- and goal-tracking behaviors in male and female rats. Mean +SEM PavCA Index of **A-C**) females and **D-F**) males trained in conjunction with females, as well as **C**) females and **F**) males split by phenotype during the last session of PavCA acquisition (Session 5) and two additional sessions (Session 6 -7) on which treatment was reversed. For female rats **A**) previously receiving CORT and switched to VEH, there was a significant Effect of Session (p=0.039). Female rats showed an increase in sign-tracking behavior when CORT was no longer administered. For female rats split by phenotype **C**) there was an Effect of Session (p<0.001), Phenotype (p<0.001), and Session x Phenotype interaction (p<0.001) for those who previously received VEH. Female goal-tracker rats showed a significant increase in sign-tracking behavior with the administration of CORT. Session 7 is significantly different from session 5 (p=0.007) and 6 (p<0.001). No significant effects were observed in males.

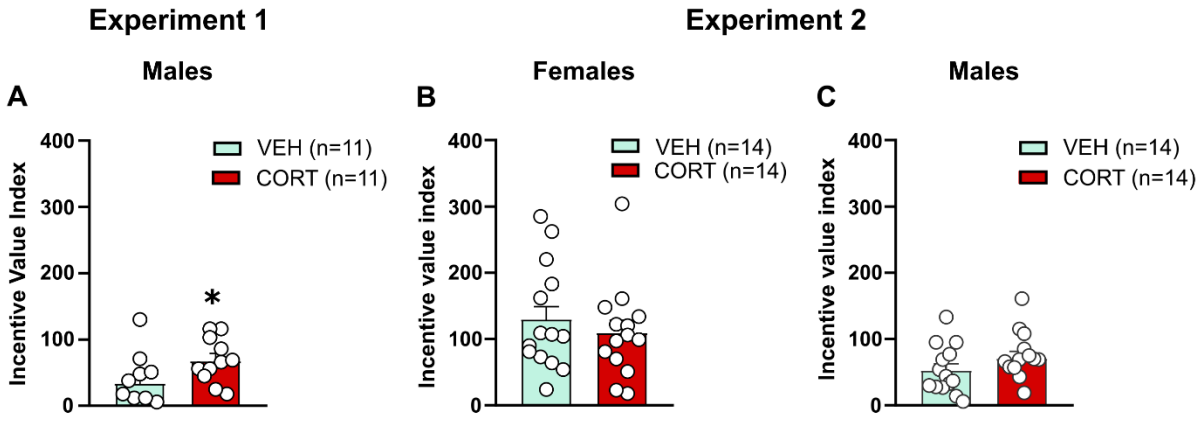

Figure 3.4 Conditioned reinforcement test (CRT). Mean + SEM incentive value index for **A**) Experiment 1 males, **B**) Experiment 2 females, and **C**) males trained in conjunction with females. Incentive value index is only significant in **A**) males trained separately from females (*p*=0.044), with rats previously administered CORT exhibiting a greater incentive value index.

Acquisition PavCA Behavior

Experiment 1 Males

Sign-tracking	Lever Contacts			Lever Contact Probability			Lever Contact Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 21	6.254	p=0.021	1, 21.957	5.049	p=0.035	1, 21.158	6.448	p=0.019
Effect of Session	4, 21	8.155	p<0.001	4, 44.069	12.005	p<0.001	4, 33.129	8.282	p<0.001
Treatment*Session	4, 21	2.648	p=0.062	4, 44.069	3.999	p=0.017	4, 33.129	2.777	p=0.043

Goal-tracking	Food cup Entries			Food cup Entry Probability			Food cup Entry Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 24.924	3.373	p=0.078	1, 31.067	3.576	p=0.068	1, 35.637	3.174	p=0.083
Effect of Session	4, 78.960	0.455	p=0.768	4, 43.353	0.271	p=0.895	4, 48.236	0.400	p=0.808
Treatment*Session	4, 78.960	0.811	p=0.522	4, 43.352	0.323	p=0.861	4, 48.236	0.426	p=0.789

Experiment 2 Females vs. Males

Sign-tracking	Lever Contacts			Lever Contact Probability			Lever Contact Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 115	3.582	p=0.061	1, 116.21	3.520	p=0.063	1, 115	3.913	p=0.050
Effect of Sex	1, 115	6.582	p=0.012	1, 116.21	8.648	p=0.004	1, 115	8.432	p=0.004
Effect of Session	4, 115	26.149	p<0.001	4, 227.40	34.301	p<0.001	4, 115	38.522	p<0.001
Treatment*Sex	1, 115	0.731	p=0.394	1, 116.21	0.697	p=0.404	1, 115	1.347	p=0.248
Treatment*Session	4, 115	2.270	p=0.066	4, 227.40	0.667	p=0.615	4, 115	0.777	p=0.542
Treatment*Sex* Session	4, 115	4.32	p=0.785	4, 227.40	0.949	p=0.436	4, 115	0.847	p=0.498

Goal-tracking	Food cup Entries			Food cup Entry Probability			Food cup Entry Latency		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 118.56	0.761	p=0.385	1, 115	2.059	p=0.154	1, 118.44	1.289	p=0.259
Effect of Sex	1, 118.56	11.529	p=0.001	1, 115	4.497	p=0.036	1, 118.44	5.048	p=0.027
Effect of Session	4, 221.65	5.209	p<0.001	4, 115	7.303	p<0.001	4, 202.53	8.019	p<0.001
Treatment*Sex	1, 118.56	0.133	p=0.716	1, 115	0.238	p=0.627	1, 118.44	0.111	p=0.740
Treatment*Session	4, 221.65	2.300	p=0.060	4, 115	2.173	p=0.076	4, 202.53	2.490	p=0.044
Treatment*Sex* Session	4, 221.65	0.962	p=0.429	4, 115	0.941	p=0.443	4, 202.53	0.906	p=0.462

Table 3.1 Linear mixed model analysis for sign- and goal-tracking behaviors. Effect of Treatment, Session, and Treatment x Session interactions were analyzed for Experiment 1 (top). Effect of Treatment, Sex, Session, Treatment x Sex, Treatment x Session, and Treatment x Sex x Session interactions were analyzed for Experiment 2 (bottom).

Acquisition PavCA Behavior

Experiment 1
Males

	Sign-tracking Lever Contacts					Goal-tracking Food cup Entries				
	1	2	3	4	5	1	2	3	4	5
VEH vs. CORT	p=0.250	p=0.158	p=0.024	p=0.019	p=0.008	p=0.888	p=0.360	p=0.130	p=0.039*	p=0.024
	Lever Contact Probability					Food cup Entry Probability				
	1	2	3	4	5	1	2	3	4	5
VEH vs. CORT	p=0.806	p=0.314	p=0.012*	p=0.034*	p=0.024*	p=0.664	p=0.226	p=0.178	p=0.095	p=0.037
	Lever Contact Latency					Food cup Entry Latency				
	1	2	3	4	5	1	2	3	4	5
VEH vs. CORT	p=0.447	p=0.218	p=0.016*	p=0.015*	p=0.013*	p=0.665	p=0.272	p=0.367	p=0.095	p=0.038

Experiment 2
Females vs. Males

	Sign-tracking Lever Contacts					Goal-tracking Food cup Entries				
	1	2	3	4	5	1	2	3	4	5
VEH vs. CORT	p=0.039	p=0.037	p=0.052	p=0.083	p=0.324	p=0.268	p=0.931	p=0.077	p=0.158	p=0.460
Female VEH vs. CORT	p=0.006	p=0.026	p=0.115	p=0.115	p=0.220	p=0.580	p=0.818	p=0.157	p=0.388	p=0.259
Male VEH vs. CORT	p=0.909	p=0.476	p=0.238	p=0.377	p=0.870	p=0.309	p=0.914	p=0.273	p=0.255	p=0.928
	Lever Contact Probability					Food cup Entry Probability				
	1	2	3	4	5	1	2	3	4	5
VEH vs. CORT	p=0.132	p=0.080	p=0.043	p=0.144	p=0.141	p=0.752	p=0.762	p=0.025	p=0.068	p=0.274
Female VEH vs. CORT	p=0.027	p=0.039	p=0.127	p=0.199	p=0.121	p=0.981	p=0.982	p=0.077	p=0.123	p=0.106
Male VEH vs. CORT	p=0.912	p=0.687	p=0.178	p=0.432	p=0.598	p=0.671	p=0.650	p=0.158	p=0.297	p=0.934
	Lever Contact Latency					Food cup Entry Latency				
	1	2	3	4	5	1	2	3	4	5
VEH vs. CORT	p=0.079	p=0.051	p=0.035	p=0.112	p=0.162	p=0.440	p=0.754	p=0.047*	p=0.069	p=0.417
Female VEH vs. CORT	p=0.006	p=0.010	p=0.049	p=0.134	p=0.140	p=0.574	p=0.908	p=0.117	p=0.130	p=0.167
Male VEH vs. CORT	p=0.741	p=0.887	p=0.309	p=0.454	p=0.620	p=0.595	p=0.575	p=0.210	p=0.287	p=0.806

Table 3.2 Treatment comparisons. Bonferroni post hoc comparisons between treatment for each PavCA session and sex are reported. Sign-tracking and goal-tracking behaviors are included for Experiment 1 (top) and Experiment 2 (bottom). *p<0.005

Conditioned Reinforcement Test

Experiment 1 Males

	Nosepokes			Lever Contacts		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 40	4.651	p=0.037	1, 20	5.739	p=0.027
Effect of Noseport	1, 40	2.869	p=0.098			
Treatment*Noseport	1, 40	0.010	p=0.916			

Experiment 2 Females

	Nosepokes			Lever Contacts		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 54	1.154	p=0.288	1, 26	0.661	p=0.424
Effect of Noseport	1, 54	44.660	p<0.001			
Treatment*Noseport	1, 54	0.077	p=0.782			

Experiment 2 Males

	Nosepokes			Lever Contacts		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 50	8.679	p=0.005	1, 26	0.008	p=0.930
Effect of Noseport	1, 50	58.571	p<0.001			
Treatment*Noseport	1, 50	5.059	p=0.029			

Table 3.3 Univariate analysis of variance for conditioned reinforcement test (CRT). Nose pokes and total lever contacts were assessed. Effect of Treatment, Noseport, and Treatment x Noseport interaction were analyzed for Experiment 1 (top) and Experiment 2 (bottom).

Chapter 4

Elucidating the Relationship Between Corticosterone and Dopamine in the Nucleus Accumbens Shell of Male and Female Sign-tracker and Goal-tracker Rats

Abstract

The goal-tracker/ sign-tracker animal model can be exploited to better understand the psychological and neurobiological processes involved in predictive vs. incentive cue-reward learning. This model has prompted us to redefine the role of dopamine (DA) in reward-learning, demonstrating that dopamine is necessary for the development of a sign-tracking, and not goal-tracking conditioned response. Thus, identifying systems that interact with DA to promote incentive learning may help understand individual variation in the propensity to attribute incentive value to cues. Glucocorticoids (GCs), or corticosterone (CORT) in rodents, have long been known to interact with DA within the nucleus accumbens (NAc) to potentiate motivated behaviors. While the NAc is a critical brain structure for incentive learning, we lack an understanding of the role of CORT in this regard. The current study aimed to probe the relationship between DA and CORT within the NAc of female and male goal- and sign-tracking rats. To date, it has been demonstrated that, relative to goal-trackers, male sign-trackers have an enhanced plasma CORT response to a single session of Pavlovian conditioning, before the development of a conditioned response.

Here, we assessed both central (i.e., NAc) and peripheral (i.e., plasma) levels of CORT over the course of Pavlovian cue-reward learning. That is, prior to and following the development of a conditioned response. We found that elevated DA levels within the NAcS are unique to sign-trackers, relative to goal-trackers, and that DA and DA-metabolites consistently differ throughout the development of a conditioned response between phenotypes. Although we found no evidence of a significant relationship between CORT and DA in the NAc of goal- or sign-trackers, these studies revealed that patterns of CORT over the course of Pavlovian cue-reward learning are dependent on sex and phenotype.

Introduction

Predictive and incentive cue-reward learning are psychologically (for review, see Berridge, 2001) and neurobiologically dissociable (e.g., Campus et al., 2019; Flagel, Clark, et al., 2011; Pitchers et al., 2017). Using the goal-tracker/sign-tracker animal model, we have been able to identify brain regions and circuits that promote predictive learning vs. incentive learning (for review see Kuhn, 2018). Rats that primarily utilize a predictive learning strategy, goal-trackers (GTs), are believed to rely on “top-down” cortical control (Campus et al., 2019; Sarter & Phillips, 2018). In contrast, rats that use both a predictive and incentive learning strategy - sign-trackers (STs) - rely on sub-cortical “bottom-up” processes (Flagel, Cameron, et al., 2011; Haight et al., 2020; Haight et al., 2017; Yager et al., 2015). Indeed, this animal model allowed us to address a long-standing debate in the field, surrounding the role of subcortical dopamine (DA) in cue-reward learning. That is, whether dopamine was central to mediating predictive (Schultz et al., 1997) vs. incentive learning (Berridge & Robinson, 1998). It was

demonstrated that phasic DA release within the nucleus accumbens core (NAcC) encodes incentive value, as the shift in DA release from the reward to the cue occurs in STs, and not GTs (Flagel, Clark, et al., 2011). Further, systemic and intra NAcC administration of a non-selective DA receptor antagonist revealed that DA is necessary for the acquisition of a sign-tracking response, or the attribution of incentive value, but not the development of a goal-tracking response. These studies established that the mesolimbic DA system encodes the incentive value of reward cues, and not the predictive value, as was the prevailing view. However, as we continue to elucidate the neural circuits and identify the neurobiological systems that play a role in incentive motivational processes, additional questions have emerged regarding the role of DA in cue-reward learning. For example, identifying systems that interact with DA to promote or suppress DA-dependent learning (e.g, Campus et al., 2019; Haight et al., 2020).

For more than twenty years, the interaction between glucocorticoid (GC) levels and DA release within the NAc has been implicated in individual differences in drug-taking behaviors (Piazza & Le Moal, 1996). Specifically, stressors (i.e., a state of elevated GC levels) (e.g., Abercrombie et al., 1989) and administration of GCs were found to increase extracellular levels of DA (e.g., Piazza et al., 1996), and this positive GC-DA relationship was thought to increase the reinforcing properties of drugs and, in turn, potentiate drug-seeking behavior (Piazza & Le Moal, 1998). Further, GCs have been shown to have reinforcing properties of their own, as rats willingly self-administer corticosterone (CORT), the main GC in rodents (Deroche et al., 1993; Piazza et al., 1993). This effect is also thought to rely on CORT-DA interactions (for review see Piazza & Le Moal, 1997), and it has been suggested that these interactions occur in a

state-dependent manner. That is, in situations when DA transmission is more engaged or elevated, as is the case during eating or in individuals with altered dopamine activity (e.g., high-responder rats) (Piazza et al., 1996). These studies prompted the hypothesis that CORT and DA interact to regulate the propensity to attribute incentive value to reward cues (Lopez & Flagel, 2020).

The goal of the current study was to probe the relationship between CORT and DA within the NAc shell (NAcS) of male and female rats across the course of Pavlovian conditioned approach (PavCA) training. DA within this subregion of the NAc has been shown to be more responsive to stressors (e.g., Kalivas & Duffy, 1995), adrenalectomy (e.g.,Barrot et al., 2000), glucocorticoid receptor (GR) antagonist (Marinelli et al., 1998), and has been recently implicated in goal-tracking vs. sign-tracking behaviors in male rats (Campus et al., 2019; Saddoris et al., 2015). Thus, the NAcS could be a prime point of interaction between CORT and DA in cue-reward learning.

Additionally, to date, our understanding of CORT levels between goal- and sign-tracker rats comes from a single study assessing plasma CORT levels early in training, prior to and immediately following the first training session (Flagel et al., 2009). It was demonstrated that CORT levels rise to a greater extent in male sign-trackers, relative to goal-trackers, even before the development of a conditioned response (Flagel et al., 2009). Here, we aimed to expand these findings by assessing plasma CORT pre/post session 1 in male and female rats, and to compare these measures with those captured later in training (pre/post session 6), once a conditioned response was acquired. Together, DA and both central (i.e., NAcS) and peripheral measures of CORT were determined over the course of predictive vs. incentive learning in male and female rats.

Our results highlight the unique role of DA in the development incentive learning, suggesting differences between sexes, and revealing sex- and phenotype-dependent differences in central and plasma CORT.

Materials and Methods

Subjects

A total of thirty female and thirty male Sprague-Dawley rats, with weights between 225-275 g, arrived from Charles River (colony R08 and R09, Raleigh, NC, USA) and Taconic Biosciences (colony BU016, Cambridge City, IN, USA) across three experimental rounds. Upon arrival, rats were paired-housed with the same sex in standard acrylic cages (46 x 24 x 22 cm) in a temperature-controlled (22 ± 2°C) housing room with a 12 hr light: dark cycle (lights on at 06:00 or 07:00 depending on daylight savings time) for the duration of the study. All rats had *ab libitum* access to food and water.

Rats remained undisturbed and were allowed to acclimate for seven days after arrival. Following the acclimation period, rats underwent surgery for implantation of a unilateral guide-cannula and allowed seven days of recovery. Rats were single-housed following surgery and remained so for the duration of the study. After recovery from surgery, rats were handled briefly for four consecutive days. They were transported to a white-lit room that was designated for tail-nicks and handled one at a time to habituate them to the room and experimenter. Once all rats were handled they were returned to the housing room. The last two days of handling, rats received twenty-five 45-mg banana-flavored grain pellets (Bio-Serv, Flemington, NJ, USA) inside the home cage, to habituate them to the food reward used during Pavlovian conditioned approach training.

All experimental procedures took place during the light phase of the cycle (between 09:00 to 17:00). Procedures followed *The Guide for the Care of and Use of Laboratory Animals: Eighth Edition* standards (2011, National Academy of Sciences) and were approved by the University of Michigan Institutional Animal Care and Use Committee.

Surgical procedures

Rats were anesthetized using 5% inhaled isoflurane (Isothesia - Butler-Schein, Columbus, OH) and maintained with 2% isofurane. Rats were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA or Stoelting, Wood Dale, IL), and once the skull was secured, they were given a subcutaneous injection of Carprofen (5 mg/kg) for analgesia. The shaved scalp was sanitized with betadine (Purdue Products, Stamford, CT), and 70% alcohol prep pads (COVIDIEN Curity) three times before being excised to expose the skull. A small hole was unilaterally drilled above the nucleus accumbens shell (NAcS), and a guide-cannula was implanted (CMA 12, Harvard Apparatus, Holliston, MA) +1.7 mm AP; ±0.8 mm ML; -0.6 mm DV from bregma, with the left and right hemisphere counterbalanced. Stainless steel screws and dental acrylic cement (Bosworth New Truliner, Keystone Industries, Gibbstown, NJ) were used to secure the cannula to the skull. Wire stylets (i.e., "dummy cannula") were placed in the guide cannula to prevent clogging.

Tail nicks and blood sampling

This study was designed to determine if we could replicate prior findings reported by Flagel et al., (2009), demonstrating a rise in CORT following the initial Pavlovian training session, and to a greater extent in sign-tracker rats; and to examine whether this pattern held true after a conditioned response is established. Thus, unlike Chapter

2, in which we assessed only baseline levels of CORT, here we performed a total of four lateral tail vein nicks per rat (two nicks a day) to assess “pre-“ vs. “post-“ session levels. Tail nicks were performed before and after the first (Session 1) and last (Session 6) session of Pavlovian conditioning (refer to experimental timeline, Figure 4.1). Baseline nicks were performed two-hours post-lights on (starting 09:00 h). Rats were taken one at a time, from the housing room to the designated baseline tail-nick room, under white light conditions. Lighting conditions were not altered (i.e. to red light) as we did not want to perturb baseline circadian levels of CORT. An experimenter lightly restrained each rat under a blue pad near the edge of a flat surface, allowing their tail to hang off. A second experimenter performed a tail nick (~ 5 mm) with the tip of a razor blade and collected blood via capillary action into a plastic tube with an EDTA coating (FisherScientific Catalog No. NC9141704). Rats were returned to the housing room and were left undisturbed for two hours prior to additional experimental procedures. Post-session tail nicks were performed as described above, with the exception of taking place in the behavior room instead of the designated baseline tail nick room. Rats were nicked as soon as their training session concluded, with their microdialysis probe still attached. An experimenter lightly restrained the rat against their body while the second experimenter performed the brief nick and blood extraction under red lighting. Samples were immediately capped and placed onto ice. The entire process of blood collection was performed within 90 secs. Samples were centrifuged at 13,000 RPM at 4° C for 10 min. ~ 40µL of plasma was extracted, flash-frozen, and stored at -20° C until radioimmunoassay.

Monitoring the estrous cycle

Identical to Chapter 3, female rats were monitored daily (~ 16:00 h) for stage of estrous cycle, by vaginal lavages, for at least five days before Pavlovian conditioning and for the duration of the study. The tip of a glass eyedropper filled with sterile 0.9% saline was inserted into the vaginal opening and gentle pulsations, via slight bulb pressure, were used to extract cells. Each sample was placed into a well (of a 24-well plate), and the dropper was rinsed thoroughly with distilled water three times between each rat. Vaginal epithelial cell cytology of each sample was observed under an inverted light microscope (20X). Estrous cycle stage characterization was determined based on the following criteria (see Alonso-Caraballo & Ferrario, 2019; Hubscher et al., 2005): *(M) Metestrus*- a mix of leukocytes, cornified cells, and round cells, *(D*) *Diestrus*- mainly leukocytes and some nucleated epithelial cells*, (P) Proestrus* – mainly nucleated epithelial cells that form sheets, *(E) Estrus*- large cornified cells lacking nuclei. Body weight was tracked daily as rats were expected to be at their heaviest during *Diestrus* and their lightest during *Estrus*. Males were also weighed daily. Given the longitudinal design of the study, it was difficult to assess and identify effects of the estrous cycle on the behavioral outcome measures. Thus, these data are not shown here.

Behavioral testing

Pavlovian conditioned approach

Similar to the two previous chapters, PavCA training took place in standard test chambers (MED Associates, St. Albans, VT, USA; 20.5 × 24.1 cm floor area, 29.2 cm high) enclosed in sound-attenuating boxes. A ventilation fan provided constant air circulation and served as white noise. Each chamber was arranged with a food-cup centered on one of the 20.5 cm walls. Food-cup entries were detected by breaks of an

infra-red beam. A retractable lever was located either to the right or the left of the food-cup and became illuminated when presented. Lever contacts were detected when a force of at least 10 g was used to deflect it. On the opposite 20.5 cm wall, a white house light was placed 1 cm from the top of the chamber. House light illumination signaled the beginning of the session and remained on for the duration of the session. A swiveled arm with a hanging spring leash was sitting directly above the chamber. A circular opening allowed the leash to come into the chamber for full motion. All behavioral testing was performed under red light conditions.

Rats underwent one pre-training session. The food-cup was baited prior to the session with three grain-pellets to direct the rats' attention to the location of reward. Once tethered and placed in the chamber rats were allowed to acclimate for 5 min before the house light turned on to signal the beginning of the session. The session consisted of 25 trials during which the lever remained retracted, and pellets were delivered randomly into the food-cup; one pellet per trial on a variable interval 30 s schedule (range 0-60 s). The total session length was approximately 12.5 min.

Twenty-four hours following pre-training, rats began PavCA training, which was conducted over 6 consecutive daily sessions. Each session consisted of 25 trials on a variable interval 90 s schedule (VI 90, range 30-150 s) during which the illuminated lever (conditioned stimulus, CS) was presented for a total of 8 s, and immediately upon its retraction, a food pellet (unconditioned stimulus, US) was delivered into the adjacent food-cup. Each session lasted approximately 40 min.

The following behavioral measures were recorded during each PavCA session: (1) number of lever contacts, (2) latency to contact the lever for the first time, (3)

probability to contact the lever, (4) number of food-cup entries during presentation of the lever, (5) latency to first enter the food-cup during presentation of the lever, (6) probability of entering the food-cup during presentation of the lever, and (7) number of food-cup entries during the inter-trial interval. These values were then used to calculate three measures of approach behavior that comprise the PavCA index: (1) response bias [(total lever presses – total food-cup entries) ÷ (total lever presses + total food-cup entries)], (2) probability difference [probability to approach the lever – the probability to enter the food-cup], (3) latency difference [± (latency to approach the lever – latency to enter the food-cup) ÷ 8]. As previously described (Meyer et al., 2012), the PavCA index score was calculated using this formula: [(response bias + probability difference + latency difference) ÷ 3]. Scores ranged from +1 to -1; a more positive score indicated a preference for sign-tracking behavior and a negative score for goal-tracking. Rats were characterized as sign-trackers, goal-trackers and intermediate responders based on their average PavCA index scores from sessions 4 and 5 (Meyer et al., 2012). Unlike the previous two chapters, the cutoffs for phenotype classification were: ≤ -0.3 for a GT, ≥ 0.3 for a ST, and in between -0.3 and 0.3 for an IR.

Microdialysis

Sampling

Dialysate samples were collected on the first (Session 1) and last (Session 6) session of PavCA training. From these samples, extracellular levels of the following neurochemicals were assessed: corticosterone (CORT), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT), GABA, glutamate (Glu), glutamine (Gln). On the day of collection, rats were briefly

anesthetized with 5% isoflurane inhalation, the wire stylets were removed, and a brand new dialysis probe (2 mm membrane length, CMA12) was inserted through the guide cannula to reach the nucleus accumbens shell (NAcS). Rats were placed inside the chamber and left undisturbed for 120 min (similar to Campus et al., 2019) with artificial cerebral spinal fluid (aCSF, PH 7.4) perfusing at a rate of 1.3 μl/ min. The filtered aCSF perfusate medium consisted of a 145 mM NaCl, 2.68 mM KCl, 1.40 $CaCl_2$, 1.01 mM $MgSO_4$, 1.55 mM Na_2HPO_4 (dibasic), 0.45 mM NaH_2PO_4 (monobasic), and 0.25 mM ascorbic acid diluted to 1:1000. Baseline samples were collected every 10 min for 60 min, and session samples collected every 10 min for the duration of the PavCA training session (~40 min). Samples (total volume of 13 μl) were further divided into 3 μl, for DA, DOPAC, HVA, 3-MT, GABA, Glu, Gln detection, and 10 μl samples, for CORT. The 3 μl samples were derivatized as described below. All samples, including the 10 μl, were flash-frozen with dry ice, where they remained for the duration of the microdialysis procedures, and then stored at -80 °C until they were analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) or radioimmunoassay.

Benzoyl chloride derivatization

Benzoyl chloride derivatization of internal standards, calibration standards, and dialysate samples was performed similar to (Wong et al., 2016). Calibration curves were generated using 0 (aCSF-only blank), 0.1, 0.5, 1, 5, 10, and 20 nM for DA. Derivatization was executed in a 2:1:1:1 ratio. For each 3 μl sample, the following was added: 1.5 μl of 0.1M sodium carbonate buffer, 1.5 μl of benzoyl chloride (2% in acetonitrile v/v), 1.5 μl Internal Standard (IS) mixture (245 μl H_2O, 245 μl acetonitrile,

5 μl Sulfuric acid, and 5 μl derivatized IS stock). Following each addition, samples were vortexed and tightly capped when complete.

HPLC-MS

HPLC-MS procedures and peak quantification were performed by the Kennedy Lab at the University of Michigan. Briefly, the neurochemical separation was achieved by a Thermo Fisher (Waltham, MA) Vanquish UHPLC system, automatically injecting 5 μl of the derivatized dialysate onto a Phenomenex Kinetex C18 HPLC column (2.1 mm x 100 mm, 1.7 μm). Mobile phase A consisted of 10 mM ammonium formate and 0.15% (v/v) formic acid in HPLC water. Mobile phase B was acetonitrile. DA, DOPAC, HVA, 3-MT, GABA, Glu, and Gln were detected using positive electrospray ionization (ESI) with a Thermo Fisher TSQ Quantum Ultra triple-quadrupole mass spectrometer operating in multiple-reaction monitoring mode. Automated peak integration was completed with Thermo XCalibur Quan Browser 2.1.

Radioimmunoassay

Plasma and brain CORT levels were measured using commercially available CORT I^{125} Double Antibody Radioimmunoassay (RIA) kits (MP Biomedicals, Solon, OH). Similar to Chapter 2, the manufacturer's protocol was followed verbatim. A range of 25-1000 ng/mL, for plasma samples, or a diluted range of 0.2-50 ng/mL, for dialysate samples, was used to generate a standard logarithmic curve for every set of 76 test tubes (the centrifuge test tube capacity for one spin), A total of 157 plasma samples (not including duplicates or calibration standards) and 516 dialysate samples (not including standards) were assayed over 18 centrifuge spins across 6 days, with no more than 4 sets (i.e., centrifuge spins) per day. Gamma radiation counts per minute, averaged across

duplicates for plasma samples and single values for dialysate samples, were converted into CORT concentrations using the average standard curve generated from all sets that were run for each day of RIA. On average, the intra-assay coefficient of variation for plasma samples was 7.78%, while, the inter-assay coefficient of variation was 19.67% for plasma samples and 60.72% for dialysate samples. Outliers were identified and removed if: 1) duplicates had a percent error greater than 10%, 2) dialysate CORT concentrations were outside the 0.1-50 ng/mL range, 3) microdialysis probe placement was off target, or 4) samples were identified as an extreme outlier (3x the interquartile range) by statistical software.

Histology

To determine probe placement within the NAcS brain slices (2.76-0.96 mm from bregma) were mounted on glass slides, stained with Cresyl-violet (Sigma-Aldrich, St.Louis, MO), and covered-slipped. A Leica DM 1000 light microscope was used to verify placement, with the experimenter blind to phenotype and sex. Data included here are from the forty-one rats for which neurochemical levels have been obtained, and are classified as GTs or STs. It should be noted, however, that probe placement verification is ongoing for thirteen of these rats. Two rats have been removed thus far, one for poor placement and another for unusual enlarged ventricles.

Statistical Analysis

As we were interested in only assessing the extreme phenotypes, STs and GTs, IRs (n= 12) were excluded from graphical representations and analysis. PavCA behavior, plasma CORT, and nucleus accumbens neurochemical extracellular levels were analyzed using the Statistical Package for the Social Sciences (SPSS) program

version 26.0 (IBM, Armok, NY, USA). Linear mixed-effects models were performed using the best fit covariance structure with the lowest Akaike's information criterion for each data set. Statistical significance was set at $p<0.05$ and Bonferroni post hoc analysis were conducted when significant interactions were detected. Neurochemical outliers (3x the interquartile range) detected by SPSS were excluded from analysis. All behavior, plasma CORT, and neurochemical levels figures were made using GraphPad Prism 8. Additional analysis comparing the relationship between neurochemicals and phenotypes were performed by Dr. Liza Levina's research group in the Department of Statistics at The University of Michigan. Wilcoxon two-sample tests comparing neurochemical profiles of GTs vs. STs were performed using R Studio version 1.3.1. Correlations were performed using Python version 3.8.3 and pairwise deletion was used to account for missing values. Statistical significance was set at $p<0.05$. Correlation heatmaps were generated with Python.

Results

Pavlovian conditioned approach (PavCA) behavior

Across six consecutive PavCA sessions, lever- (i.e., sign-tracking) and food-cup- (i.e., goal-tracking) directed behaviors were compared between female and male goal-tracker and sign-tracker rats (female GTs (n=5), STs (n= 19); male GTs (n=7), STs (n=11)). Female and male rats acquired lever-directed behaviors similarly. There was an Effect of Session, Phenotype, and a Session x Phenotype interaction, for all lever-directed behaviors including, probability [Effect of Session: $F_{5,38.11}=13.908$, $p<0.001$; Effect of Phenotype: $F_{1,38.067}=138.979$, $p<0.001$; Session x Phenotype interaction: $F_{5,38.11}=17.957$, $p<0.001$] (Figure 4.2 A), total number of contacts [Effect of Session:

$F_{5,37.739}$=7.063, p<0.001; Effect of Phenotype: $F_{1,37.528}$=59.48, p<0.001; Session x Phenotype interaction: $F_{5,37.739}$=6.949, p<0.001] (Figure 4,2C), and latency to approach the lever-CS [Effect of Session: $F_{5,64.598}$=15.245, p<0.001; Effect of Phenotype: $F_{1,41.18}$=93.426, p<0.001; Session x Phenotype interaction: $F_{5,64.598}$=17.839, p<0.001] (Figure 4.2E). Phenotype differences were apparent as early as the first conditioning session. Female and male rats differed in food-cup-directed behaviors, with females having a higher probability [Effect of Sex: $F_{1,37.901}$=5.356, p=0.026] (Figure 4.2B), greater number of contacts [Effect of Sex: $F_{1,43.475}$=7.022, p=0.011] (Figure 4.2D), and lower latency to approach the food-cup [Effect of Sex: $F_{1,42.645}$=7.466, p=0.009] (Figure 4.2F), relative to males. There was an Effect of Phenotype and a Session x Phenotype interaction for probability [Effect of Phenotype: $F_{1,37.901}$=13.846, p=0.001; Session x Phenotype interaction: $F_{5,38.062}$=20.946, p<0.001], number of entries [Effect of Phenotype: $F_{1,43.475}$=18.56, p<0.001; Session x Phenotype interaction: $F_{5,77.63}$=18.334, p<0.001, and latency [Effect of Phenotype: $F_{1,42.645}$=18.302, p<0.001; Session x Phenotype interaction: $F_{5,59.295}$=22.988, p<0.001 to enter the food-cup, with Phenotype differences as early as the third conditioning session.

Plasma Corticosterone (CORT)

Plasma CORT levels of female and male sign- and goal-tracker rats were assessed at baseline (i.e., pre) and immediately following (i.e., post) the first (Figure 4.3B) and last (Figure 4.3C) PavCA session. Plasma CORT levels increased from pre- ($\bar{x}$=105.88 ng/mL) to post-session ($\bar{x}$=289.48 ng/mL) [Effect of Timepoint: $F_{1,24.898}$=25.266, p<0.001] as a function of Sex and Phenotype [Timepoint x Sex x Phenotype: $F_{1,24.828}$=5.966, p=0.022] (Figure 4.3A). That is, plasma CORT levels

increased from pre- to post-session only for female goal-trackers (n=4) and male sign-trackers (n=7). Additionally, while females ($\bar{x}$=279.3 ng/mL) had higher levels of plasma CORT relative to males ($\bar{x}$=116.06 ng/mL) [Effect of Sex: $F_{1,16.625}$=8.571, p=0.010], sex differences were specific to the post-session timepoint for GTs (p= 0.011) and pre-session timepoint for STs (p= 0.021) (Figure4.3A). ST vs. GT (p=0.041) differences were only apparent for male rats, with STs ($\bar{x}$=308.97 ng/mL) having greater plasma CORT levels post-session, relative to GTs ($\bar{x}$=81.51 ng/mL) (Figure 4.3A).

Microdialysis

Corticosterone (CORT)

CORT levels within the nucleus accumbens shell of male and female sign-trackers and goal-trackers were similar under baseline conditions ($\bar{x}$=2.88 ng/mL) and during the PavCA session ($\bar{x}$=3.69 ng/mL) [Effect of Phase: $F_{1,95}$=0.756, p=0.387], and before and after the conditioned response had been acquired [Effect of Session: $F_{1,95}$=2.837, p=0.095], (Figure4.4B; Figure4.4C). Overall, female rats ($\bar{x}$=5.87 ng/mL) had greater levels of CORT compared to males ($\bar{x}$=1.28 ng/mL) [Effect of Sex: $F_{1,95}$=18.605, p<0.001] (Figure4.4A).

Dopamine and dopamine metabolites

DA levels within the nucleus accumbens shell (Figure 4.5A), increased with the development of a conditioned response [Effect of Session: $F_{1,125}$=9.552, p=0.002], from session 1 ($\bar{x}$=5.01 nM) (Figure4.5B) to session 6 ($\bar{x}$=8.60 nM) (Figure4.5C). However, DA levels during the PavCA session ($\bar{x}$=6.84 nM) were similar to those captured under baseline ($\bar{x}$=6.77 nM) across training [Effect of Phase: $F_{1,125}$=0.004, p=0.949]. Relative to GTs ($\bar{x}$=4.47 nM), STs ($\bar{x}$=9.14 nM) had higher levels of DA [Effect of Phenotype:

$F_{1,125}$=16.158, p<0.001]. There was a significant effect of sex for nucleus accumbens shell DA levels, [Effect of Sex: $F_{1,125}$=10.848, p=0.001], with males ($\bar{x}$=8.72 nM) having greater levels of DA, relative to females ($\bar{x}$=4.89 nM).

All DA metabolites, DOPAC, 3-MT, HVA, and DA metabolites to DA ratios captured the same Phenotype differences as DA, with STs having higher levels of DA metabolites and lower DA metabolite/ DA ratios (see Table 4.1). While, sex differences were only apparent for the 3-MT/DA ratio; males had lower 3MT/DA levels ($\bar{x}$=0.61 nM), relative to females ($\bar{x}$=0.78 nM). Finally, from session 1 to 6, only DOPAC, 3-MT, and DOPAC/DA ratio significantly changed (see Table 4.1).

GABA

GABA within the nucleus accumbens shell of male and female sign-trackers and goal-trackers were not significantly different [Effect of Phenotype: $F_{1,121}$=1.12, p=0.293; Effect of Sex: $F_{1,121}$=0.282, p=0.596] and did not significantly change with the development of a conditioned response [Effect of Session: $F_{1,121}$=2.94, p=0.088] (data not shown in graphical form).

Glutamate

Glutamate levels in the nucleus accumbens shell increased from session 1 ($\bar{x}$=320863.55 nM) to session 6 ($\bar{x}$=869154.45 nM) [Effect of Session: $F_{1,127}$=30.027, p<0.001] in a phenotype- [Effect of Phenotype: $F_{1,127}$=11.226, p=0.001] and sex-dependent [Effect of Sex: $F_{1,127}$=8.952, p=0.003] manner [Session x Phenotype interaction: $F_{1,127}$=9.831, p=0.002; Session x Sex interaction: $F_{1,127}$=8.354, p=0.005; Session x Phenotype x Sex interaction: $F_{1,127=}$4.965, p=0.028] (Figure 4.6). Specifically, there was a significant rise from session 1 to session 6 in nucleus accumbens shell

glutamate in female GTs (p<0.001) and female STs (p=0.031) (Figure 4.6A), and session 6 levels were significantly greater for female GTs ($\bar{x}$=1712176 nM, p<0.001), relative to female STs ($\bar{x}$=614702.15 nM).

Neurochemical correlations

The relationship between neurochemical profiles across the course of predictive- (i.e., in GTs) vs. incentive (i.e., in STs) learning was assessed, with male and female rats collapsed as these relationships do not differ between sexes (Figure 4.7A-D). Correlation coefficients and p-values are listed in Table 4.2. Significant positive relationships between dopamine (DA) and DA metabolites (HVA, DOPAC, 3-MT) were present across training and phenotypes (Figure 4.7A-D). There were, however, no significant relationships between DA and corticosterone (CORT). In GTs, there was a trend for a negative relationship between CORT and glutamate (Glu) during the first training session (r=-0.6613, p=0.052) (Figure 4.4.7A or Table 4.2A), and a significant positive relationship with GABA during the 6th PavCA session (r=0.9127, p=0.001) (Figure 4.4.7B or Table 4.2B). Whereas, in STs there appears to be no relationship between CORT and any other neurochemical detected. Preliminary correlation analysis, revealed no relationship between plasma CORT and any of the neurochemicals (data now shown).

Goal-tracker/sign-tracker differences in neurochemical profiles

Wilcoxon two-sample tests comparing neurochemical profiles of GTs and STs revealed significant differences in DA and DA metabolite levels under baseline conditions and during the first and last PavCA training sessions (see Table4.3). DA-related neurochemicals, however, were not significantly different under baseline

conditions on the last session (session 6), and CORT, Gln, Glu, and GABA were not significantly different between phenotypes at any time point.

Discussion

The goal of the present study was to determine the relationship between dopamine (DA) and corticosterone (CORT) within the nucleus accumbens shell (NAcS) of male and female goal- and sign-tracker rats. Additionally, we aimed to expand on the phenotypic differences in plasma CORT that are apparent in male rats early in cue-reward learning. We found that 1) plasma CORT levels increased following PavCA training as a function of phenotype and sex. Within the NAcS, 2) CORT levels were greater in female rats, relative to male rats, and 3) DA increased with the development of a conditioned response (from session 1 to 6). Independent of the extent of training, male rats, and sign-trackers in general had greater levels of DA. 4) We did not observe a significant relationship between DA and CORT within the NAcS.

Plasma CORT

Our hypothesis of the role of glucocorticoids in incentive learning stems from the Flagel et al. (2009) findings of a greater rise in plasma CORT levels in male sign-tracker rats, relative to goal-trackers, with initial training. However, data from Tomie et al. (2000) suggested these phenotypic differences would be apparent later in learning (e.g., following the 20th session). Here, we were able to replicate this greater rise in CORT levels for male STs, relative to male GTs, and revealed that these patterns held true regardless of being assessed early in training (session 1) or once a conditioned response was acquired (session 6). Additionally, for the first time, we assessed plasma CORT levels in female goal- and sign-trackers. We found that CORT significantly

increased in female GTs, relative to female STs. Still, levels were not significantly different from female STs, perhaps because female STs started with higher levels before the training sessions. Indeed, female STs had elevated baseline plasma CORT levels that were significantly different from those of male STs. It is not unexpected for female rats to exhibit greater levels of plasma CORT (e.g., Atkinson & Waddell, 1997). However, the discovery that these sex differences are based on phenotype and only apparent in sign-tracker rats, is novel. Taken together, these data suggest that peripheral CORT levels, both baseline and those in response to cue-reward learning, are dependent on sex and innate cue-learning strategy. Notably, baseline plasma levels did not significantly increase from session1 to 6 as observed in Chapter 2. Differences are most likely due to the use of different cohort of animals, but it should be considered that others have discussed inconsistent results, particularly the presence or lack of a relationship between CORT and behavior, dependent on the number of nicks performed (Tomie et al., 2002).

NAcS CORT

Unlike plasma CORT, CORT levels measured within the NACs did not differ based on phenotype. However, they did vary based on sex. Overall, female rats had greater CORT levels relative to males. Few studies have used the microdialysis technique to measure CORT within the brain (see Linthorst & Reul, 2008), and fewer have captured levels within the NAc in response to rewards (Keller et al., 2017; Palamarchouk et al., 2009). There are contradictory findings as to whether brain levels are synchronized with blood levels, fluctuating at an ultradian and circadian rhythm similar to plasma CORT (Droste et al., 2008; Qian et al., 2012). Here, we did not

observe a significant correlation between peripheral and central CORT. Thus, our findings are contributing to the understanding of central CORT, but perhaps the NAcS is not the primary target of CORT in this context. As few studies have assessed central CORT levels, there is little evidence for region-specific changes in CORT. It has been reported, however, using microdialysis, that under alcohol withdrawal (e.g., Dominguez et al., 2017) or chronic stress (e.g., Dominguez et al., 2019), there are changes in CORT specifically in the prefrontal cortex and not the hippocampus. Such examples suggest that cue-reward learning could potentially influence CORT concentrations in a region-specific manner, and it is possible that such effects are occurring outside of the NacS.

It is also important to note that, quantitative values, or extracellular concentrations of the neurochemical of interest (e.g., CORT or DA), captured via the microdialysis technique, are not representative of absolute baseline levels or stimulus-induced exocytotic release (for review see, Krebs-Kraft et al., 2007). Instead, concentrations analyzed from dialysate samples are levels that have not been cleared from the extracellular space. Further, methods like in-vitro (e.g., Campus et al., 2019) or in-vivo (e.g., Kawa et al., 2019) recovery are often implemented to more accurately estimate neurochemical concentrations by assessing the performance of the collection probe. While in-vitro recovery was performed for DA analysis in the current study, this is lacking for our CORT analysis and that of others in the brain (e.g., Keller et al., 2017; Palamarchouk et al., 2009). Thus, CORT data are often presented as percent change from baseline and this should be considered when interpreting our CORT concentration values. Notably, however, others have reliably measured CORT within the brain and

implemented probe recovery (e.g.,Linthorst et al., 1995). Thus, we do not believe that recovery data would significantly impact the results or interpretation of our CORT analyses.

NAcS DA

This study is the first, to our knowledge, to assess extracellular DA levels across the course of PavCA training (during sessions 1 and 6) in GTs vs. STs, and the first to compare male and female DA levels in this context. While DA levels increased with cue-reward learning experience and were greater in males and sign-trackers, we did not capture a rise in DA levels during the conditioning sessions, relative to baseline conditions. Techniques with a different temporal resolution, like fast-scan cyclic voltammetry (FSCV), have previously revealed increases in phasic DA within the NAc core in response to the cue and the reward (Flagel, Clark, et al., 2011). The possibility that DA changes are perhaps too rapid to be captured by microdialysis methods should be considered. However, a study measuring extracellular DA within NAcS of male sign-tracker and goal-tracker rats suggests that DA levels do increase during the 6th PavCA training session (Campus et al., 2019). Notably, collection frequency is half the time implemented in our experiment (5 vs. 10 min). Further, microdialysis methods can influence whether DA levels reflecting “physiological neurotransmitter release” are successfully and accurately measured (for review, see Robinson & Camp, 1991). For example, the acclimation period following probe insertion can be an important factor. We have reason to believe (see Appendix B), however, that the acclimation period used in this study allowed us to capture functionally relevant DA levels; as we determined that DA levels measured using a 2-hr acclimation period were sensitive to Ca^{2+} changes

in perfusate medium (see Appendix B). Finally, while there are advantages for repeated collection from the same site in a given animal, differences in DA levels have been reported with multiple probe insertions that may prevent stimulus-induced changes (e.g., amphetamine, K^+) (Camp & Robinson, 1992; Robinson & Camp, 1991). These differences are often a decrease in DA metabolite levels. Given we saw an increase in DA and 3-MT, and no change in HVA from session 1 (1st probe insertion) to session 6 (2nd probe insertion) we believe it is unlikely that multiple probe insertions interfered with DA levels. We did however, capture a decrease in DOPAC. Interestingly, phenotype comparisons of all neurochemical profiles revealed that DA and DA-metabolites are consistently and significantly different between GTs and STs across training. In summary, our findings continue to highlight the DA system as key determinant of goal- vs. sign-tracking.

NAcS CORT-DA interaction

There was no significant relationship between CORT and DA within the NAcS.. One explanation regarding the lack of a CORT-DA interaction, is the state-dependent effect of CORT on DA. Some have failed to see this interaction when assessed during the light-cycle in rodents (e.g., Imperato et al., 1991). Further, in this regard, the CORT-DA relationship might be in place only when the CORT system is significantly altered. For example, in response to stress (e.g., Abercrombie et al., 1989; Hall et al., 1998; Kalivas & Duffy, 1995), with administration of high levels of CORT (Piazza et al., 1996), and in rodents with altered CORT (Piazza, Maccari, et al., 1991) and/or DA (Piazza, Rouge-Pont, et al., 1991) systems (i.e., high responders) (Piazza et al., 1996). In humans, for example, altered glucocorticoids are captured once a biologically disruptive

experience has occurred. For example, with history of drug-taking (Contoreggi et al., 2003) or following trauma (Delahanty et al., 2000). Similarly, such experiences (e.g., drug-administration or extreme stress) have been shown to alter the glucocorticoid system of rodents (Sarnyai et al., 1993; Whitaker & Gilpin, 2015). It is possible, therefore, that only under such conditions would CORT and DA be primed to interact with one another to mediate reward learning.

Additionally, the NAcS could perhaps not be the point of intersection of CORT-DA, that is relevant to incentive learning. Other brain structures, including the prefrontal cortex, where this hormone is believed to mediate DA efflux (Butts et al., 2011), may be an important locus of interaction (Pitchers et al., 2017) and should be investigated further in future studies. Finally, it appears that, in GTs, CORT activity is correlated with that of glutamate and GABA throughout the course of learning, whereas these relationships are not apparent in STs. Thus, CORT maybe be interacting with other neurotransmitters within the NAcS to influence cue-reward learning in a phenotype-dependent manner.

Conclusion

In conclusion, while peripheral CORT levels differ between goal- and sign-trackers in male rats, these distinct patterns are not evident in female rats. For the first time we assessed CORT and DA extracellular levels within the NAcS throughout the course of predictive vs. incentive learning. While we were unable to uncover a relationship between these molecules, we report that DA activity within the NAcS (including that of its metabolites) is especially relevant to the sign-tracking response. These findings will serve as the foundation for future research surrounding the role of

CORT-DA interactions in goal- vs. sign-tracking behaviors. In particular, these findings suggest that future studies should focus on this interaction when the CORT system is disrupted or stimulated, as is often the case in psychopathology.

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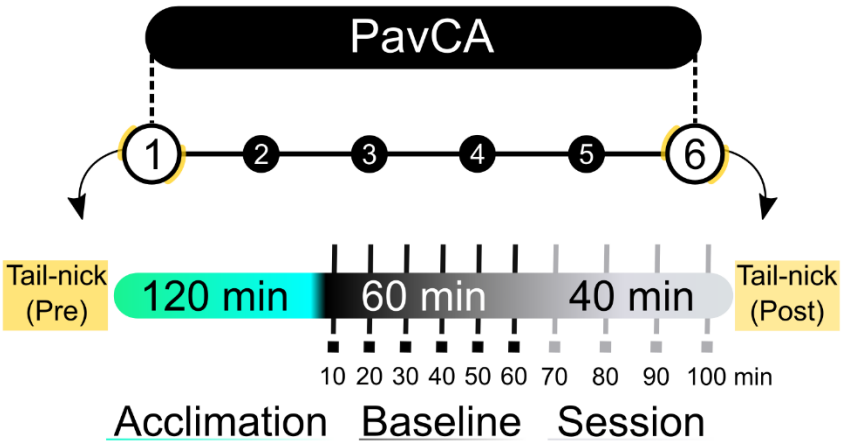

Figure 4.1 Experimental timeline. On sessions 1 (i.e., first session) and 6 (i.e., last session) of Pavlovian conditioned approach (PavCA) training: plasma corticosterone (CORT) levels were assessed via lateral tail-nicks pre- and post-session; and CORT and dopamine within the nucleus accumbens shell (NAcS) were assessed via microdialysis methods. Once probes were inserted to reach the NAcS, rats were allowed a 120 min period for acclimation. Dialysate samples were collected every 10 min for a 60-min baseline period and during the 40-min PavCA session.

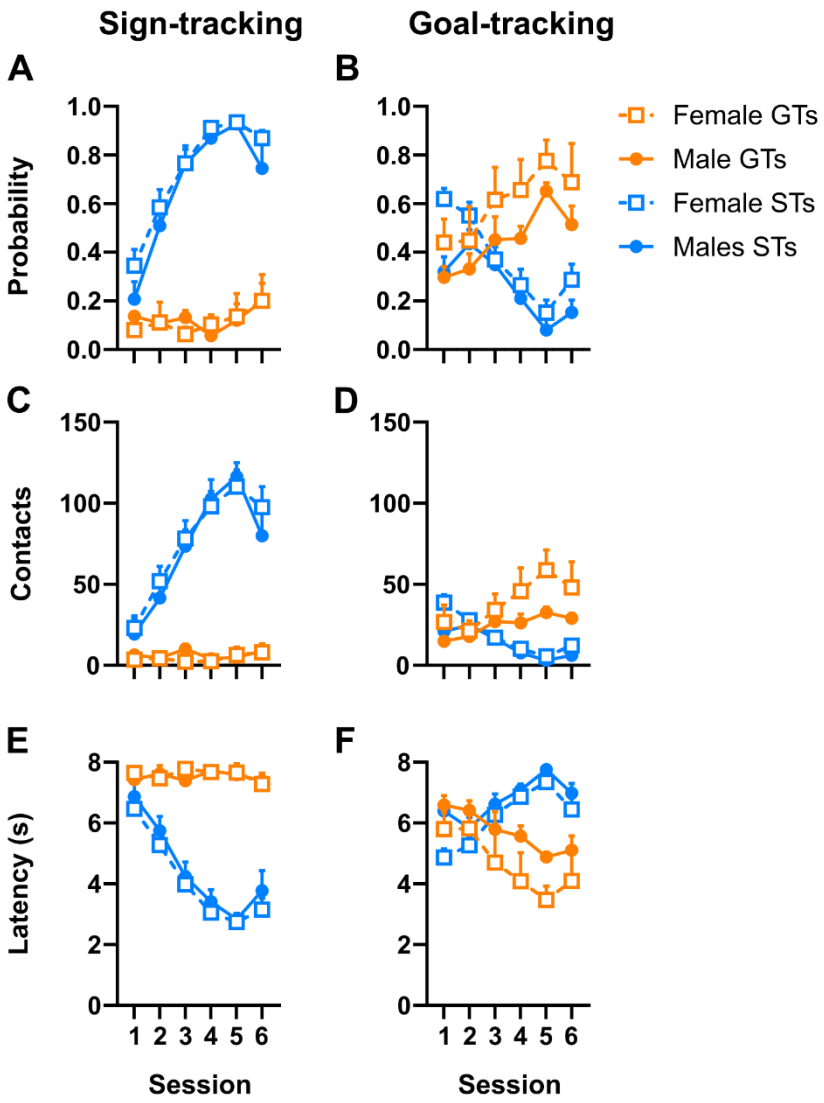

Figure 4.2 Acquisition of sign-tracking and goal-tracking behavior for male and female rats. Lever-directed behaviors (i.e., sign-tracking) and food-cup directed behaviors (i.e., goal-tracking) were assessed across 6 PavCA training sessions. Mean + SEM for probability to **A**) contact the lever or **B**) enter the food-cup, total number of contacts with **C**) the lever or **D**) the food-cup, and latency to **E**) contact the lever or **F**) enter the food-cup during the presentation of the lever-cs. Male (n= 18) and female (n= 24) rats were characterized as goal-trackers (female GTs (n=5), male GTs (n=7)) and sign-trackers (female STs (n=19), male STs (n=11)).

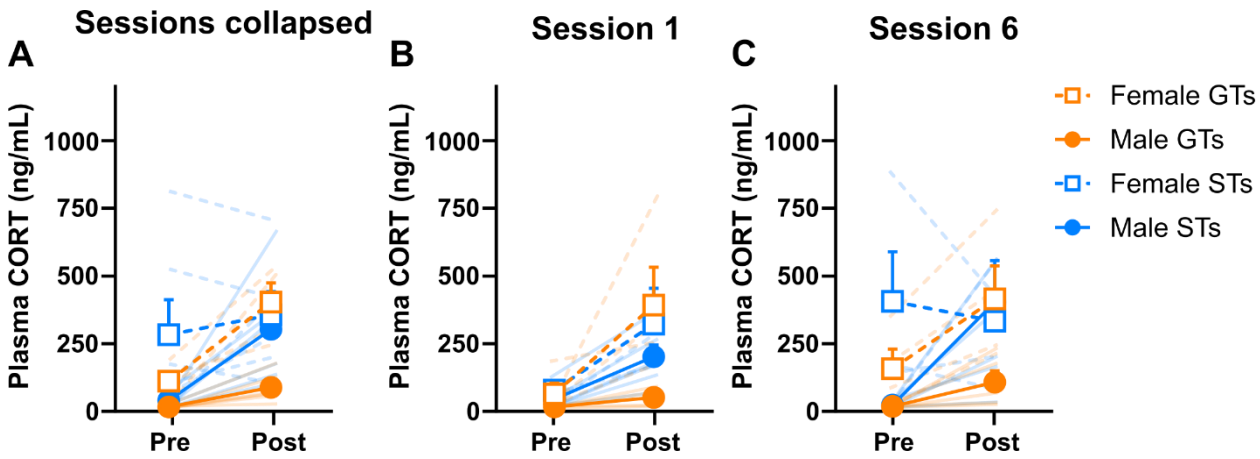

Figure 4.3 Plasma corticosterone (CORT) levels of male and female goal-tracker and sign-tracker rats. Single values (lighter opacity lines) and mean + SEM (connected data points) for plasma CORT levels measured pre- and post- **A**) Sessions 1 and 6 collapsed, **B**) Session 1, and **C**) Session 6 of PavCA training. CORT levels were not significantly different between **B**) Session 1 and **C**) Session 6 ($p = 0.059$). **A**) Collapsed across both sessions of PavCA training, CORT levels significantly differed from pre to post ($p < 0.001$). Post hoc analysis revealed CORT rises for female goal-trackers (orange open squares, $p = 0.001$) and male sign-trackers (blue solid circles, $p = 0.001$). Phenotype differences are only apparent in males (GTs vs. STs, $p = 0.041$) immediately following the session (post). Sex differences were apparent based on Phenotype and Timepoint ($p = 0.022$). Relative to male STs (blue solid circles), female STs (blue open squares, $p = 0.011$) had greater levels of CORT pre-session, while relative to male GTs (orange solid circles), female GTs (orange open squares, $p = 0.021$) had greater CORT levels post-session. Female GTS (n=4), Male GTs (n=6), Female STs (n=6), Male GTs (n=7).

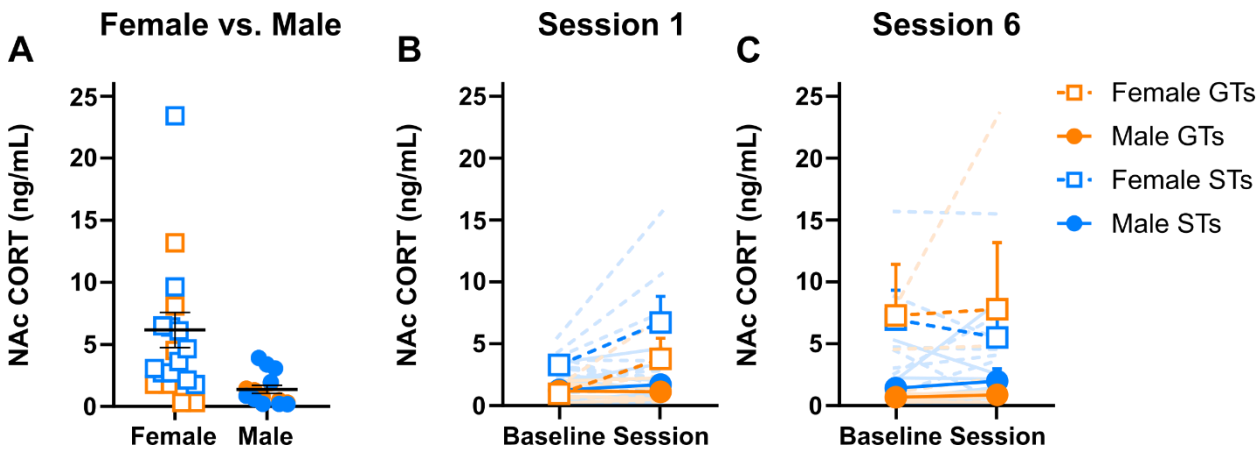

Figure 4.4 Corticosterone (CORT) levels within the nucleus accumbens (NAc) shell of male and female goal-tracker and sign-tracker rats. A) Mean + SEM for NAc CORT levels collapsed across phase (Baseline vs. Session) and PavCA sessions (Session 1 vs. 6) for female and male rats. Overall, female rats (open squares, p <0.001) had higher levels of CORT compared to males (solid circles). **B-C**) Single values (lighter opacity lines) and mean + SEM (connected data points) for NAc CORT levels measured under baseline conditions and during PavCA **B**) Session 1 and **C**) Session 6. CORT levels captured during the PavCA session did not significantly differ from baseline (p = 0.387) and were similar between **B**) Session 1 and **C**) Session 6 (p =0.095). Female GTs (n=5), Male GTs (n=7), Female STs (n=10), Male STs (n=9).

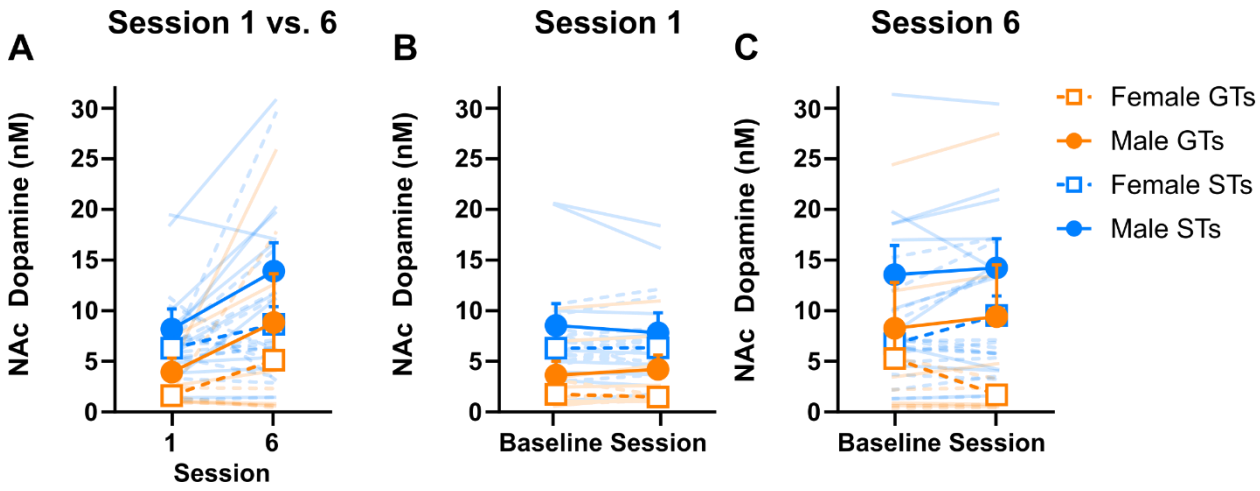

Figure 4.5 Dopamine (DA) levels within the nucleus accumbens (NAc) shell of male and female goal-tracker and sign-tracker rats. Single values (lighter opacity lines) and mean + SEM (connected data points) for NAc shell DA levels **A**) collapsed across phase (Baseline vs. Session) and compared between Sessions 1 vs. 6; and under baseline conditions vs. during PavCA **B**) Session 1, **C**) Session 6. DA levels captured during the PavCA session did not significantly differ relative to baseline levels (p = 0.945). **A**) DA levels increased with the development of a conditioned response, from **B**) Session 1 to **C**) Session 6 (p =0.002) of PavCA training. Overall, sign-tracker rats (in blue, p <0.001) had higher levels of DA compared to GTs (in orange). Relative to female rats (open squares), males (solid circles) had higher DA levels (p =0.001). Female GTs (n=5), Males GTs (n=7), Female STs (n=17), Male STs (n=10).

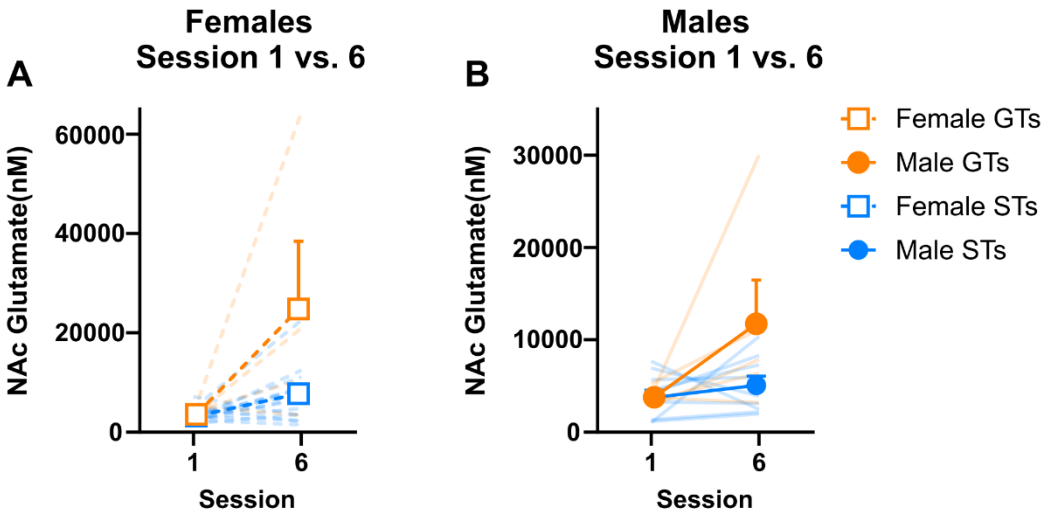

Figure 4.6 Glutamate levels within the nucleus accumbens (NAc) shell of female and male goal-tracker and sign-tracker rats. Single values (lighter opacity lines) and mean + SEM (connected data points) for NAc shell glutamate levels collapsed across phase (Baseline vs. Session) and compared between Sessions 1 vs. 6 for **A**) female and **B**) male rats. Glutamate levels increased with the development of a conditioned response, from Session 1 to Session 6 (p<0.00). Post hoc analysis revealed an increase for female GTs (orange open squares, p<0.001) and female STs (blue open squares, p =0.013), with female GTs having greater glutamate levels compared to female STs (p<0.001). Female GTs (n=5), Male GTs (n=7), Female STs (n=16), Male STs (n=10).

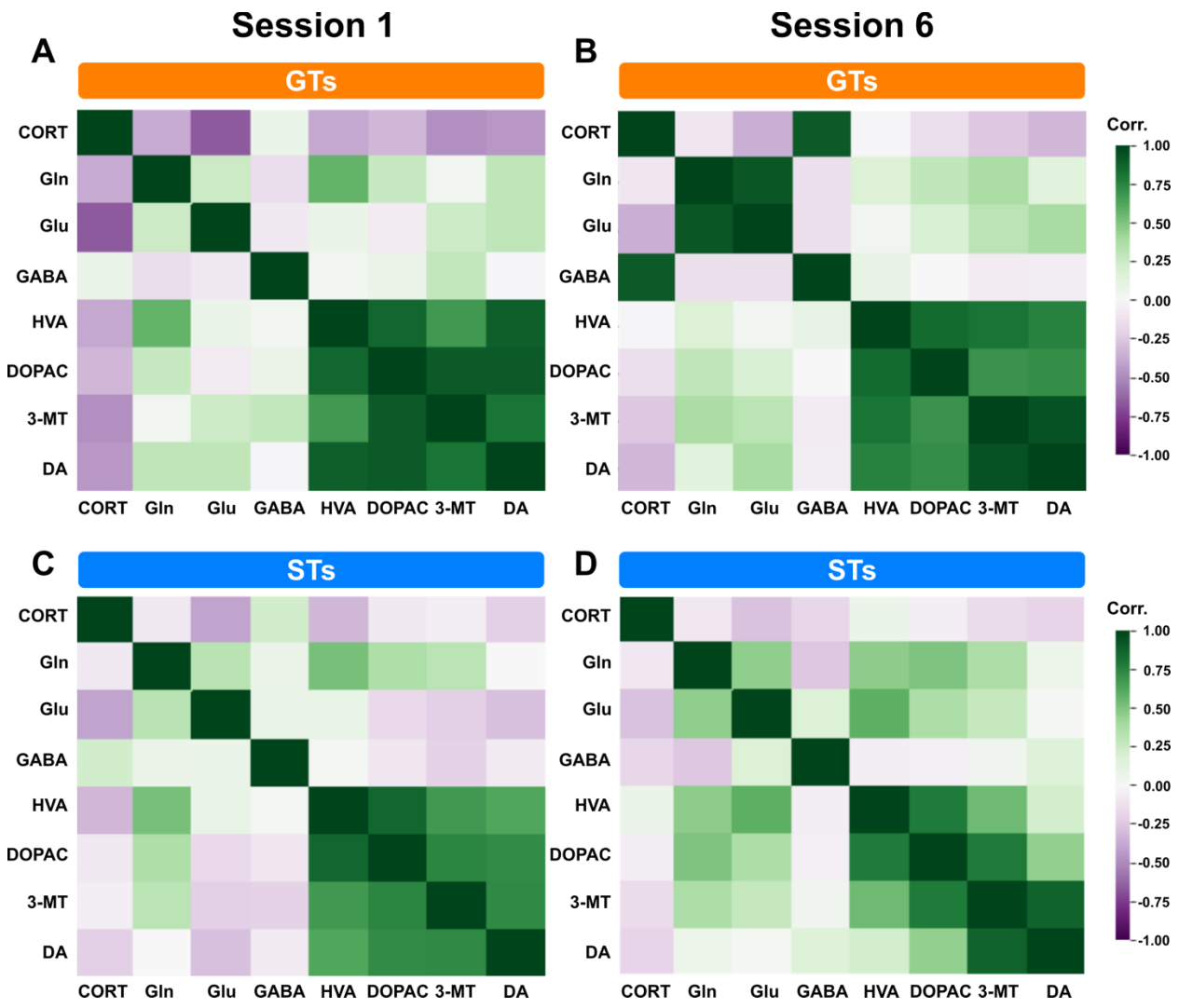

Figure 4.7 The relationship between neurochemicals within the nucleus accumbens of goal- and sign-tracker rats during Pavlovian conditioning. Heatmaps representing the direction, green (+)/purple (-), and strength (i.e., color intensity) of the correlations (r-values) between different neurochemicals within the nucleus accumbens shell of **A-B**) goal-trackers (GTs) and **C-D**) sign-trackers (STs) during the **A, C**) first and **B, D**) last PavCA training session. Corticosterone (CORT), glutamine (Gln), glutamate (Glu), GABA, homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), dopamine (DA).

Dopamine metabolites	Session 1 vs. Session 6	Baseline vs. Session	Males vs. Female	Goal-trackers vs. Sign-trackers
DOPAC	$F_{1,128}$=6.602, p=0.011*****	$F_{1,128}$=0.051, p=0.822	$F_{1,128}$=3.056, p=0.083	$F_{1,128}$=26.826, p<0.001*****
3-MT	$F_{1,124}$=8.121, p=0.005*****	$F_{1,124}$=0.007, p=0.934	$F_{1,124}$=1.946, p=0.166	$F_{1,124}$=28.963, p<0.001*****
HVA	$F_{1,127}$=0.544, p=0.462	$F_{1,127}$=1.697, p=0.195	$F_{1,127}$=2.481, p=0.118	$F_{1,127}$=14.942, p<0.001*****
DOPAC/DA ratio	$F_{1,128}$=7.865, p=0.006*****	$F_{1,128}$=0.762, p=0.384	$F_{1,128}$=0.168, p=0.683	$F_{1,128}$=30.534, p<0.001*****
3-MT/DA ratio	$F_{1,125}$=1.131, p=0.290	$F_{1,125}$=0.396, p=0.530	$F_{1,125}$=9.766, p=0.002*****	$F_{1,125}$=5.673, p=0.019*****
HVA/DA ratio	$F_{1,125}$=0.579, p=0.448	$F_{1,125}$=1.366, p=0.245	$F_{1,125}$=0.003, p=0.960	$F_{1,125}$=47.167, p<0.001*****

Table 4.1 Results from Linear mixed model analysis for dopamine metabolites and dopamine metabolite to dopamine ratios. Effect of Session, Phase, Sex, Phenotype were analyzed. DOPAC, 3-MT, HVA, DOPAC/DA, 3-MT/DA, and HVA/DA ratios significantly differed based on Phenotype, with STs having greater metabolite levels and lower metabolite to dopamine ratios, relative to GTs. *****, p<0.005.

A) Goal-trackers: Session 1

	CORT	GLN	GLU	GABA	HVA	DOPAC	3-MT	DA
CORT	r=1, p<0.001*	r=-0.3718, p=0.3245	r=-0.6613, p=0.0524	r=0.079, p=0.8663	r=-0.3834, p=0.3085	r=-0.3245, p=0.3942	r=-0.4767, p=0.2324	r=-0.4504, p=0.2628
GLN	r=-0.3718, p=0.3245	r=1, p<0.001*	r=0.2548, p=0.4242	r=-0.1438, p=0.6919	r=0.5715, p=0.0523	r=0.2693, p=0.3973	r=0.0445, p=0.8966	r=0.3017, p=0.3672
GLU	r=-0.6613, p=0.0524	r=0.2548, p=0.4242	r=1, p<0.001*	r=-0.0834, p=0.8189	r=0.0808, p=0.8028	r=-0.0661, p=0.8383	r=0.2476 p=0.4628	r=0.3032, p=0.3648
GABA	r=0.079, p=0.8663	r=-0.1438, p=0.6919	r=-0.0834, p=0.8189	r=1, p<0.001*	r=0.0368, p=0.9195	r=0.0706, p=0.8462	r=0.2835, p=0.4598	r=-0.0216, p=0.9561
HVA	r=-0.3834, p=0.3085	r=0.5715, p=0.0523	r=0.0808, p=0.8028	r=0.0368, p=0.9195	r=1, p<0.001*	r=0.8629, p=0.0003*	r=0.6758, p=0.0224*	r=0.8926, p=0.0002*
DOPAC	r=-0.3245, p=0.3942	r=0.2693, p=0.3973	r=-0.0661, p=0.8383	r=0.0706, p=0.8462	r=0.8629, p=0.0003*	r=1, p<0.001*	r=0.9109, p=0.0001*	r=0.9073, p=0.0001*
3-MT	r=-0.4767, p=0.2324	r=0.0445, p=0.8966	r=0.2476, p=0.4628	r=0.2835, p=0.4598	r=0.6758, p=0.0224*	r=0.9109, p=0.0001*	r=1, p<0.001*	r=0.8103, p=0.0025*
DA	r=-0.4504, p=0.2628	r=0.3017, p=0.3672	r=0.3032, p=0.3648	r=-0.0216, p=0.9561	r=0.8926, p=0.0002*	r=0.9073, p=0.0001*	r=0.8103, p=0.0025*	r=1, p<0.001*

B) Goal-trackers: Session 6

	CORT	GLN	GLU	GABA	HVA	DOPAC	3-MT	DA
CORT	r=1, p<0.001*	r=-0.0988, p=0.8159	r=-0.3524, p=0.4932	r=0.9127, p=0.0016*	r=-0.0232, p=0.9564	r=-0.1381, p=0.7443	r=-0.251, p=0.5487	r=-0.3229, p=0.4354
GLN	r=-0.0988, p=0.8159	r=1, p<0.001*	r=0.924, p=0.001*	r=-0.1256, p=0.7474	r=0.1726, p=0.6335	r=0.2895, p=0.4171	r=0.3778, p=0.2817	r=0.1473, p=0.6847
GLU	r=-0.3524, p=0.4932	r=0.924, p=0.001*	r=1, p<0.001*	r=-0.1285, p=0.7837	r=0.0461, p=0.9136	r=0.1947, p=0.644	r=0.3136, p=0.4495	r=0.3863, p=0.3445
GABA	r=0.9127, p=0.0016*	r=-0.1256, P=0.7474	r=-0.1285, p=0.7837	r=1, p<0.001*	r=0.1049, p=0.7883	r=0.0021, p=0.9958	r=-0.0691, p=0.8597	r=-0.0603, p=0.8775
HVA	r=-0.0232, p=0.9564	r=0.1726, p=0.6335	r=0.0461, p=0.9136	r=0.1049, p=0.7883	r=1, p<0.001*	r=0.8465, p=0.002	r=0.8056, p=0.0049*	r=0.7589, p=0.0109*
DOPAC	r=-0.1381, p=0.7443	r=0.2895, p=0.4171	r=0.1947, p=0.644	r=0.0021, p=0.9958	r=0.8465, p=0.002	r=1, p<0.001*	r=0.698, p=0.0248*	r=0.7132, p=0.0206*
3-MT	r=-0.251, p=0.5487	r=0.3778, p=0.2817	r=0.3136 p=0.4495	r=-0.0691, p=0.8597	r=0.8056 p=0.0049*	r=0.698 p=0.0248*	r=1, p<0.001*	r=0.9449, P<0.001*
DA	r=-0.3229, p=0.4354	r=0.1473, p=0.6847	r=0.3863, p=0.3445	r=-0.0603, p=0.8775	r=0.7589, p=0.0109*	r=0.7132, p=0.0206*	r=0.9449, P<0.001*	r=1, p<0.001*

C) Sign-trackers: Session 1

	CORT	GLN	GLU	GABA	HVA	DOPAC	3-MT	DA
CORT	r=1, p<0.001*	r=-0.0804, p=.7434	r=-0.4055, p=0.1063	r=0.2333, p=0.3514	r=-0.3274, p=0.1713	r=-0.0835, p=0.734	r=-0.0485, p=0.8534	r=-0.2181, p=0.3698
GLN	r=-0.0804, p=0.7434	r=1, p<0.001*	r=0.326, p=0.1201	r=0.0731, p=0.7285	r=0.5187, p=0.0066*	r=0.3619, p=0.0692	r=0.3172, p=0.1309	r=0.0062, p=0.9759
GLU	r=-0.4055, p=0.1063	r=0.326, p=0.1201	r=1, p<0.001*	r=0.0829, p=0.7069	r=0.0969, p=0.6524	r=-0.1715, p=0.4231	r=-0.2137, p=0.3395	r=-0.288, p=0.1724
GABA	r=0.2333, p=0.3514	r=0.0731, p=0.7285	r=0.0829, p=0.7069	r=1, p<0.001*	r=0.0167, p=0.9367	r=-0.1012, p=6301	r=-0.2082, p=0.3404	r=-0.0737, p=0.7264
HVA	r=-0.3274, p=0.1713	r=0.5187, p=0.0066	r=0.0969, p=0.6524	r=0.0167, p=0.9367	r=1, p<0.001*	r=0.863, p<0.001*	r=0.6777, P=0.0003*	r=0.6328,p= 0.0005*
DOPAC	r=-0.0835, p=0.734	r=0.3619, p=0.0692	r=-0.1715, p=0.4231	r=-0.1012, p=0.6301	r=0.863, P<0.001*	r=1, p<0.001*	r=0.7484, p<0.001*	r=0.7229, p=0.001*
3-MT	r=-0.0485, p=0.8534	r=0.3172, p=0.1309	r=-0.2137, p=0.3395	r=-0.2082, p=0.3404	r=0.6777, p=0.0003*	r=0.7484, P<0.001*	r=1, p<0.001*	r=0.7329, p=0.001*
DA	r=-0.2181, p=0.3698	r=0.0062, p=0.9759	r=-0.288, p=0.1724	r=-0.0737, p=0.7264	r=0.6328, p=0.0005*	r=0.7229, P<0.001*	r=0.7329, p<0.001*	r=1, p<0.001*

D) Sign-trackers: Session 6

	CORT	GLN	GLU	GABA	HVA	DOPAC	3-MT	DA
CORT	r=1, p<0.001*	r=-0.0862, p=0.7421	r=-0.2736, p=0.2879	r=-0.1813, p=0.4716	r=0.0789, p=0.7556	r=-0.0518, p=0.8384	r=-0.1529, p=0.5446	r=-0.2006, p=0.44
GLN	r=-0.0862, p=0.7421	r=1, p<0.001*	r=0.4557, p=0.0252*	r=-0.2539, p=0.2313	r=0.4635, p=0.0225*	r=0.5013, p=0.0126*	r=0.3744, p=0.0714	r=0.0642, p=0.7709
GLU	r=-0.2736, p=0.2879	r=0.4557, p=0.0252*	r=1, p<0.001*	r=0.175, p=0.4133	r=0.5894, p=0.0024*	r=0.3698, p=0.0753	r=0.2751, p=0.1932	r=0.0196, p=0.9293
GABA	r=-0.1813, p=0.4716	r=-0.2539, p=0.2313	r=0.175, p=0.4133	r=1, p<0.001*	r=-0.0531, p=0.8011	r=-0.0407., p=0.847	r=0.0502, p=0.8117	r=-0.1707, p=0.4251
HVA	r=-0.0789, p=0.7556	r=0.4635, p=0.0225*	r=0.5894, p=0.0024*	r=-0.0531, p=0.8011	r=1, p<0.001*	r=0.789, P<0.001*	r=0.5433, p=0.005*	r=0.2217, p=0.2978*
DOPAC	r=-0.0518, p=0.8384	r=0.5013, p=0.0126*	r=0.3698, p=0.0753	r=-0.0407, p=0.847	r=0.789, P<0.001*	r=1, p<0.001*	r=0.7825, P<0.001*	r=0.4474, p=0.0284*
3-MT	r=-0.1529, p=0.5446	r=0.3744, p=0.0714	r=0.2751, p=0.1932	r=0.0502, p=0.8117	r=0.5433, p=0.005*	r=0.7825, P<0.001*	r=1, p<0.001*	r=0.8779, P<0.001*
DA	r=-0.2006, p=0.44	r=0.0642, p=0.7709	r=0.0196, p=0.9293	r=1707, p=0.4251	r=0.2217, p=0.2978*	r=0.4474, p=0.0284*	r=0.8779, P<0.001*	r=1, p<0.001*

Table 4.2 Results for correlation analysis of nucleus accumbens neurochemicals during Pavlovian conditioning. Correlation coefficient and significance for correlations between neurochemical levels of all goal-tracker rats during **A**) session 1 and **B**) session 6 of PavCA training, and all sign-trackers during **C**) session 1 and **D**) session 6.

	Session 1		Session 6	
	Baseline	**Session**	**Baseline**	**Session**
CORT	p=0.053875	p=0.169291	p=0.899685	p=0.287174
Gln	p=0.375959	p=0.279829	p=0.158827	p=0.384312
Glu	p=0.524985	p= 0.111784	p=0.113449	p=0.063609
GABA	p=0.653274	p=1	p=0.187952	p=0.591614
HVA	p=0.052546	p=0.170496	p=0.082823	p=0.003594*****
DOPAC	p=0.006663*****	p=0.012115*****	p=0.005094	p=0.002331*****
3-MT	p=0.000228*****	p=0.000863*****	p=0.059207	p=0.020069*****
DA	p=0.002325*****	p=0.003133*****	p=0.078665	p=0.009624*****

Table 4.3 Results for Wilcoxon two-sample tests between neurochemical profiles of goal- and sign-trackers. p-values for phenotype comparisons of neurochemical levels captured within the nucleus accumbens during session 1 and session 6 baseline and session microdialysis collections. Dopamine (DA) and DA-metabolites (HVA, DOPAC, 3-MT) were the only neurochemicals significantly different between phenotypes throughout the course of training, on session 1 and 6.

Chapter 5
General Discussion

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This dissertation sought to increase our understanding of the role of glucocorticoids in cue-reward learning. The overarching hypothesis is that glucocorticoids are involved in dopamine-dependent incentive learning, a psychological process that can transform neutral cues in the environment into powerful "lures" of aberrant behavior; and that they do so by interacting with dopamine. Neuroendocrine, pharmacological, neurochemical, and behavioral approaches were applied to address this hypothesis. Collectively, results from the series of studies performed point to a role for glucocorticoids in cue-reward learning, beyond negative valence systems. Novel findings regarding glucocorticoids (central and peripheral) and dopamine over the course of learning a cue-reward association are revealed. Direct evidence of a glucocorticoid-dopamine relationship, however, was not captured. Results from each approach are described in detail in the respective Chapters, 2-4. This chapter includes a discussion summarizing major findings as related to the literature from which we derived the proposed glucocorticoid-dopamine interaction in incentive cue-reward learning framework.

Summary of findings

Plasma corticosterone in Pavlovian conditioning

The first piece of evidence of the role of corticosterone (CORT), the main glucocorticoid in rodents, in mediating individual differences in the propensity to attribute incentive value to reward cues was reported in Flagel et al. (2009). These data demonstrated that male rats have an elevated plasma CORT response following a single session of Pavlovian conditioning (25 cue-reward pairings). Rats that eventually developed a sign-tracking response to the cue, not yet apparent in this session, had enhanced post-session plasma CORT levels, relative to rats that developed goal-tracking. Differences early in training, before the development of a conditioned response, suggested plasma CORT levels could serve as a predictor of future behaviors. Additionally, it prompted the examination of plasma CORT measures across multiple windows of cue-learning. In Chapter 2, we specifically asked whether baseline plasma CORT levels would change due to cue-reward learning. While it was suggested by Tomie et al. (2000) that baseline CORT levels would not be different between phenotypes even later in training, as they were not early in training (Flagel et al., 2009), baseline levels had not been systemically assessed in the same rat, as we did in Chapter 2. Here, we found that, indeed, baseline profiles change once a conditioned response was acquired, but not as a function of phenotype. Despite the lack of phenotypic difference in baseline plasma CORT concentrations, we analyzed the correlation between baseline levels and behaviors, but the results failed to support our hypothesis that baseline plasma CORT levels would be a predictor of behavior (Chapter 2). We did, however, find that the trajectory in cue-directed behaviors, captured by differences in behavior from the beginning to the end of the training, significantly

correlated with "ending" baseline plasma CORT profiles. This relationship between plasma CORT levels and behavior is further supported by the phenotype-dependent plasma CORT differences *following* Pavlovian conditioning (Flagel et al., 2009); and by Tomie et al. (2000) who showed that the averaged acquisition behavior (from session 1-10) positively correlated with plasma CORT levels *following* a 20th conditioning session. Together, it became evident that plasma CORT concentration differences between phenotypes would only be captured immediately following Pavlovian conditioning (Flagel et al., 2009). Thus, in Chapter 4, we aimed to replicate these initial findings (Flagel et al., 2009) in male rats and further expand our understanding of plasma CORT levels in females and males later in training, once a conditioned response was acquired. We found that the phenotypic differences in male rats remained, independent of the number of training sessions, with male sign-trackers having a greater increase in plasma CORT, relative to male goal-trackers. There was a significant rise in female goal-trackers, independent of the number of training sessions, but levels were not significantly greater than those of female sign-trackers. Thus, the phenotypic differences are only present in male rats. Female sign-trackers had exceptionally high levels of plasma CORT in this study, and baseline levels in females were significantly different from those in male sign-trackers. Higher levels of CORT in females are not unexpected (Atkinson & Waddell, 1997); however, the discovery that sex differences were dependent on phenotype, and higher baseline plasma CORT levels were only apparent in female sign-trackers and not female goal-trackers, was novel. In summary, differences in plasma CORT response to Pavlovian conditioning play a role in the propensity to goal- and sign-track to a reward cue, but distinctively between sexes.

Plasma corticosterone associated with negative valence systems

We know that glucocorticoids are not synonymous with "stress" (MacDougall-Shackleton et al., 2019) and that they have a multidimensional role across the body and brain (see General introduction, or Lopez & Flagel, 2020). However, given its central role in regulating the stress response (Herman et al., 2016) and our lack of understanding of differences in negative valence systems (RDoC) of goal-trackers vs. sign-trackers, Chapter 2 aimed to determine if there were differences between phenotypes in neuroendocrine and behavioral measures of negative valence. In male mice, it had been previously reported that sign-trackers have stress hyper-sensitivity, in that their plasma CORT response is elevated to a greater extent relative to goal-trackers in response to an acute stressor (Harb & Almeida, 2014). However, we did not find a significant difference in plasma CORT of male rats in response to an open field test or restraint stress, and no significant differences in behaviors on an elevated plus-maze or open field test (Chapter 2). We believe that discrepancies from prior results in mice are due to the species and possibly differences in the type and intensity of the stressor (Joels & Baram, 2009; Spencer & Deak, 2017). In support, Vanhille et al. (2015) did not report significant differences between sign- and goal-trackers on behavior during the elevated plus-maze test when it was conducted before acquiring a conditioned response in male rats. We did, however, find that sign-trackers have greater glucocorticoid receptor mRNA expression in the ventral hippocampus, with no phenotypic differences in the dorsal hippocampus. Given these phenotypic differences were not reflected by CORT and behavior associated with negative valence, we propose they are directly related to motivated behavior and reward learning. It is

important to note, however, that hippocampal GR mRNA may or may not directly translate to receptor expression (i.e., protein) levels. Further, as both GR and MR mRNA can be influenced by the presence or absence of glucocorticoids, the role of MR in this regard should be investigated (see Herman & Spencer, 1998), Upstream regulators and downstream effectors (i.e., functional relevance) of GR mRNA in this context also warrants further investigation. Given the current dataset, we conclude that goal-trackers and sign-trackers do not differ on behavioral or neuroendocrine measures of negative valence, and that differences in plasma CORT captured in response to Pavlovian conditioning pertain to the different cue-reward learning strategies and not to negative valence per se.

As the above studies were conducted only with male rats, it remains to be determined whether female goal-tracker and sign-tracker rats differ in negative valence systems. Female rats have been reported to show higher baseline plasma CORT levels (Kitay, 1961; Lu et al., 2015) and enhanced and prolonged response to acute and chronic stressors relative to males (Goel et al., 2014; Lu et al., 2015). Further, plasma CORT in response to stressors can differ based on the estrous cycle phase (Lu et al., 2015). Thus, while data collected from male rats allows us to make conclusions about the role of glucocorticoids beyond negative valence systems, additional studies are warranted in female rats.

Nucleus accumbens corticosterone

There is an abundance of CORT circulating at all times throughout the body, but only about ~ 5% is free from bound proteins and can enter the brain (Moisan et al., 2014). However, the amount of CORT that reaches the brain increases in response to

stimuli (e.g., stressors) (Droste et al., 2008). While the in vivo microdialysis technique has been around for more than thirty-five years (Jacobson et al., 1985; Zetterstrom et al., 1983; Zetterstrom & Ungerstedt, 1984), it wasn't until the mid-90's that brain corticosterone was reported for the first time (Linthorst et al., 1994). Most of this work has focused on measurements captured from the hippocampus and in the context of stress (e.g., Dominguez et al., 2014; Droste et al., 2008; Qian et al., 2012); but a few studies have captured this hormone in regions like the prefrontal cortex, amygdala, and nucleus accumbens in regard to reward (Keller et al., 2017; Palamarchouk et al., 2009). In Chapter 4, we assessed corticosterone levels within the nucleus accumbens shell (NAcS), in female and male goal- and sign-tracker rats. Within this brain region CORT has been reported to influence dopamine and motivated behaviors (e.g., Graf et al., 2013). As our studies represent the first to capture CORT in the nucleus accumbens shell in relation to incentive learning, the findings were informative, even if unexpected. We found that, overall, female rats have greater levels of CORT in the nucleus accumbens shell, relative to males. Levels within the accumbens, however, did not differ based on phenotype or training experience. Further, we did not observe a correlational relationship between central and peripheral CORT, when taken from the same animal. The lack of probe recovery, as expanded upon in Chapter 4 should be noted for this interpretation. Further, the synchrony of peripheral and central levels of CORT have been investigated and the results contradictory. Ultradian rhythms have been reported to be both highly synchronous (Qian et al., 2012) and distinct (Droste et al., 2008). Interestingly, a delay, from blood to brain, in response to acute stressors has been reported (Droste et al., 2008), however, this delay is argued to be due to

differences in bound vs. free plasma CORT (Qian et al., 2011). That is, when looking at free CORT, this delay is not present (Qian et al., 2011). As we relied on radioimmunoassays that detect free corticosterone (Bekhbat et al., 2018), the lack of phenotypic differences in NAcS CORT, compared to plasma, is unlikely to be explained by the bound vs. free issue. These findings suggest, therefore, that peripheral and nucleus accumbens CORT levels do not reflect the same phenotypic differences, and presumably are differentially involved in cue-reward learning.

The effect of glucocorticoid alteration on behavior

Pharmacological manipulations of glucocorticoids and Type II glucocorticoid receptors (GR) suggest that glucocorticoids are necessary for making associative memories, but this has primarily been studied in the context of aversive stimuli (e.g., Beylin & Shors, 2003; Cordero & Sandi, 1998; Lesuis et al., 2018). For example, administration of CORT (5 mg/kg) prior to Pavlovian conditioning with an aversive stimulus enhanced the magnitude of a conditioned response (Beylin & Shors, 2003). Studies using male Japanese quail, are the first to assess the effects of pharmacological manipulation of glucocorticoids on the attribution of incentive value (i.e., sign-tracking) to reward cues. Specifically, the administration of PT150, a GR antagonist, attenuates sign-tracking behavior in Japanese quail, and it does so in a dose dependent manner (Rice et al., 2018; Rice et al., 2019). Based in part on these findings, in Chapter 3 we hypothesized that a GR agonist (i.e., 3 mg/kg CORT) would enhance the propensity to sign-track. In accordance with our hypothesis we found that systemic CORT administration prior to appetitive Pavlovian conditioning promotes the *acquisition* of sign-tracking in male rats. However, the opposite was found in females,

with an attenuation of sign-tracking, that interestingly recovered once treatment was removed. Thus, CORT appears to be “suppressing” the *expression* of sign-tracking in females. Further, a reversal experimental design uncovered that goal-tracking females, were significantly responsive to the effects of CORT when administered for two consecutive days after their conditioned response had been acquired under untreated conditions. Under these conditions CORT promoted lever-directed behavior in goal-tracking females.

Notably, when trained in conjunction with females, we failed to replicate the promoting effect on sign-tracking behavior that we had initially found in male rats. We believe that, while male and female rats did not directly interact, their continuous presence may have been sufficient to influence the results (Lemaire et al., 1997), especially with the high concentration of CORT administered (Taylor et al., 1987). Together, we can conclude that systemic administration of CORT appears to influence cue-reward learning in a sex- and phenotype-dependent manner. Further these studies highlight the complexity of the role of glucocorticoids, where situational differences (i.e., presence of females), sex, and innate cue-learning strategies, dictate the influence of CORT on cue-motivated behaviors.

Nucleus accumbens shell dopamine

The dopamine system encodes the attribution of incentive value to reward cues (Flagel, Clark, et al., 2011), and the nucleus accumbens core has been identified as an important locus for the role of dopamine in incentive learning (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012). More recently, however, the role of the nucleus accumbens shell has been implicated in goal- vs. sign-tracking behaviors (Campus et

al., 2019; Flagel, Cameron, et al., 2011; Saddoris et al., 2015). In Chapter 4, we assessed extracellular levels of dopamine within the nucleus accumbens shell of male and female goal- and sign-tracker rats over the course of cue-reward learning. We found that dopamine levels increased with the development of a conditioned response, and that, independent of the conditioning session, sign-trackers and male rats have elevated levels of extracellular dopamine within the nucleus accumbens shell. Phenotypic differences were also reflected by dopamine metabolites. Comparison of dopamine and dopamine metabolites between sign-trackers and goal-trackers were consistently different across learning; whereas differences in other neurotransmitters were not as readily apparent. In summary, these data further solidify the unique role of the dopamine system in the sign-tracking response, highlighting the involvement of the nucleus accumbens shell in incentive learning and revealing novel sex differences.

One concern, regarding these studies, is that we were unable to capture a rise in dopamine during the conditioning sessions. Prior studies in our lab, using similar microdialysis methods, have successfully detected an increase of dopamine within the nucleus accumbens shell during a Pavlovian conditioning session relative to baseline (Campus et al., 2019). As reported in Appendix B, we believe that the dopamine levels measured were functional, or at least sensitive to Ca^{2+}. Further, differences between baseline and session were not captured with the first probe insertion (session 1); and dopamine and 3-MT increased, and HVA remained stable with the second probe insertion (session 6). Thus, we do not believe that multiple probe insertions influenced the reported results. Rather, it may be that the time-course of microdialysis sampling in these studies was not sufficient to capture a rise in dopamine during a session.

Discussion

Glucocorticoid-dopamine interaction

The initial link between glucocorticoids and dopamine in potentiating motivated behaviors comes from rodent studies, and specifically the high-responder/low-responder rat model. These rats are characterized based on their locomotor response to novelty and differ in reactivity to stress (Kabbaj et al., 2000; Piazza et al., 1991), dopamine transmission (Marinelli & White, 2000; Rouge-Pont et al., 1998), and susceptibility to drug-taking behaviors (Piazza et al., 1990). Differences in glucocorticoid function between these rats are believed to be related to stress-responsivity (Kabbaj et al., 2000; Piazza et al., 1991); with high-responders (HRs) exhibiting stress hyper-sensitivity, enhanced dopamine transmission, and greater susceptibility to drugs. However, HRs also show greater sensitivity to the reinforcing properties of glucocorticoids, readily self-administering low doses (Piazza et al., 1993), and exhibiting greater levels of dopamine within the nucleus accumbens in response to exogenous glucocorticoids (Piazza et al., 1996). This work established a relationship between glucocorticoid and dopamine that is relevant to reward-learning.

A proposed integrative role for glucocorticoid-dopamine interactions in determining individual differences in the propensity to attribute incentive value to reward cues

To date, the relationship between glucocorticoids and dopamine had not been probed using the goal-tracker/ sign-tracker animal model. Beyond our understanding of this relationship in the context of addiction, there was supporting data regarding incentive learning, that further merited this investigation. For example, in addition to having distinct plasma CORT levels following a single session of Pavlovian conditioning

(Flagel et al., 2009), differences in dopamine systems between sign-trackers and goal-trackers were also apparent (Flagel et al., 2007). While these two findings were not directly compared, Tomie et al. (2000) had demonstrated that a tendency for lever-directed behavior, positively correlated with elevated plasma CORT levels and tissue dopamine levels. Further, the similar attenuating effect of systemic GR- (Rice et al., 2018) and dopamine-antagonist (Flagel, Clark, et al., 2011) on sign-tracking behavior were intriguing.

In Chapter 4, the detection of glucocorticoids and dopamine from the same dialysate samples, within the nucleus accumbens shell of female and male sign- and goal-tracker rats allowed for us to assess this relationship for the first time. While we uncovered individual effects of glucocorticoids and dopamine, we failed to elucidate the relationship between the two. Interestingly, in GTs, CORT activity is correlated with that of glutamate and GABA throughout the course of learning, and these relationships are not apparent in STs. Thus, CORT maybe be interacting with other neurotransmitters within the nucleus accumbens to influence cue-reward learning in a phenotype-dependent manner.

It should be noted that the approach of microdialysis and correlation analysis has limitations. As it will be addressed within the framework below, we are unaware of the temporal-dynamics at which glucocorticoids and dopamine interact, and whether extracellular levels collected every ten minutes via microdialysis can capture this relationship. It should also be considered that the glucocorticoid-dopamine interaction has been reported to be state-dependent (e.g., in the dark-cycle) (Piazza et al., 1996), and can otherwise be masked (Imperato et al., 1991) (i.e., in the light-cycle). Thus,

further optimization of experimental design should be assessed. Finally, and further elaborated in the alternative methods section below, there are other techniques (Scheimann et al., 2019) that can offer cell- and region-specific manipulation of the glucocorticoid system that can be used to address some of these potential limitations.

Based on our findings and the overview of literature, we propose that glucocorticoids enhance dopamine transmission and, in turn, the propensity to sign-track (Figure 5.1). We know that dopamine acts specifically to encode the incentive value of reward cues, not the predictive value (Flagel, Clark, et al., 2011; Wyvell & Berridge, 2000, 2001). As the cue-reward relationship is learned, there is a shift in the dopamine response from the reward to the cue, but this shift only occurs in sign-trackers (Flagel, Clark, et al., 2011). We suspect, therefore, that glucocorticoids are acting in a time-sensitive manner to prime the dopamine system to respond selectively to the reward-cue (CS). The mechanism by which glucocorticoids and dopamine interact in this regard remains to be determined. However, given that sign-tracking behavior is dependent on dopamine in the NAc (Saunders & Robinson, 2012) we expect the primary point of intersection to be within the VTA or NAc (Figure 5.1).

The temporal dynamics of corticosterone and dopamine over the course of stimulus-reward learning should be considered within this framework. An increase in dopamine overflow within the NAc can be captured across a Pavlovian conditioning session (Campus et al., 2019); as can sub-second changes in DA in response to the reward cue (Flagel, Clark, et al., 2011). However, only a few studies have captured corticosterone levels within the NAc (e.g. Palamarchouk et al., 2009, also see Chapter 4), and the dynamics of corticosterone in this regard are largely unknown. Thus, it

remains to be determined whether the effects of GCs on stimulus-reward learning are rapid (taking seconds to minutes) and/or slow and genomic (taking hours to days) (de Kloet et al., 2008). We speculate that it is likely a combination of both. As reported above, we know that GCs rise immediately following a single Pavlovian conditioning session (Flagel et al., 2009; Tomie et al., 2002), and do so to a greater extent in STs (Flagel et al., 2009). However, pharmacological blockade of GR does not appear to have an immediate effect on sign-tracking behavior (Rice et al., 2018; Rice et al., 2019). Further, the ability of GCs to strengthen excitatory synapses of VTA-DA neurons via GR activation can take up to 2 hrs (Daftary et al., 2009; Polter & Kauer, 2014; Saal et al., 2003; Stelly et al., 2016); and stress-induced translocation of GRs to the nucleus of DA neurons occurs on a similar time scale, indicative of genomic effects (Hensleigh & Pritchard, 2013). It is possible, therefore, that activity of GCs at GR can impact behavior during a Pavlovian conditioning session, but that the interaction between GCs and dopamine that promote incentive learning rely more on genomic mechanisms.

Another potential point of intersection for glucocorticoids and dopamine within the context of incentive motivational processes, is the DAT. Interestingly, relative to GTs, STs show greater DAT surface expression in ventral striatal synaptosomes and faster dopamine uptake in the NAc (Singer et al., 2016). These findings suggest that greater DAT surface expression promotes the attribution of incentive salience to discrete reward cues (Singer et al., 2016). It is possible that glucocorticoids are playing a facilitatory role in this regard. Currently, our understanding of the mechanism by which GCs alter DAT within the NAc are limited, but are presumed to be GR-dependent and to take days (Sarnyai et al., 1998); thus, indicative of genomic effects. Importantly, while we can

learn a great deal about glucocorticoid function from the field of stress neurobiology, we recognize that the manner (genomic vs nongenomic) and mechanism by which glucocorticoids are impacting reward-processing and reward-related behaviors is likely to be quite different. We believe the use of the GT/ST animal model to investigate these relationships will increase our understanding of the role of glucocorticoids in psychopathological behaviors.

Alternative sites of glucocorticoid-dopamine interaction

Prefrontal cortex

Dopamine within the prefrontal cortex has been implicated in incentive learning (Pitchers et al., 2017). Specifically, dopamine increases in response to drug-associated cues in sign-trackers, relative to goal-trackers. Glucocorticoid receptors are also expressed within the prefrontal cortex (Herman, 1993), and have been shown to influence dopamine (Butts et al., 2011). Administration of a GR-antagonist blunts dopamine response to stress; whereas, corticosterone increases dopamine (Butts et al., 2011). Further, microdiaysis has been successfully executed to measure corticosterone within the prefrontal cortex (Palamarchouk et al., 2009). There is a rise in prefrontal CORT in response to self-administration or a non-contingent infusion of cocaine. Projections from the prefrontal cortex to the nucleus accumbens are important in mediating drug-motivated behaviors (McFarland et al., 2003). Importantly, inhibiting this pathway attenuates cue-induced relapse (Stefanik et al., 2013); and we know “top-down” control and cognitive styles are different between sign- and goal-trackers (Campus et al., 2019; Sarter & Phillips, 2018). Given the above, the prefrontal cortex

should be considered as another potential locus of interaction for dopamine and glucocorticoids in the regulation of incentive learning.

Ventral tegmental area

The ventral tegmental area (VTA) contains dopaminergic cells that project to the nucleus accumbens, where phasic dopamine (DA) release has been demonstrated to encode incentive value (Flagel, Clark, et al., 2011). The VTA-NAc pathway, specifically, has been implicated in incentive learning (e.g., Saunders et al., 2018), and more recently it has been shown, that DA neural activity within the VTA, during the presentation of the reward-cue, is enhanced in STs, those attributing incentive value to the cue to a greater degree than goal-trackers (Ferguson et al., 2020). Thus, DA cells within the VTA may be pivotal in driving individual differences in cue-learning. Interestingly, the presence of corticosterone within the VTA, is known to potentiate excitatory synapses (i.e., an increase in AMPA/NMDA ratio) on DA neurons, and it is believed to do so via glucocorticoid receptors (GRs) (e.g., Daftary et al., 2009). These plastic changes can result in firing rate alterations of DA cells projecting to brain regions like the nucleus accumbens, but also the prefrontal cortex (for review see Polter & Kauer, 2014). Thus, the VTA may play a primary role regulating DA-CORT interactions that promote cue-reward learning.

Ventral hippocampus

Alterations of the ventral hippocampus (e.g., lesions or stimulation) can influence DA levels within the nucleus accumbens and prefrontal cortex (Lipska et al., 1992; Taepavarapruk et al., 2008). Further, lesions of the ventral hippocampus, and not dorsal

hippocampus, attenuate the acquisition of sign-tracking behavior and this is accompanied by a decrease in DA metabolites, specifically HVA, and HVA/DA ratios within the nucleus accumbens (Fitzpatrick et al., 2016). From our own findings presented in Chapter 2, GR mRNA is expressed to a greater extent in the ventral hippocampus of sign-trackers, relative to goal-trackers. While the relationship between hippocampal GR and its effect on DA is unclear, we know that CORT-GR function influences glutamatergic transmission within the hippocampus (for review, see Popoli et al., 2011) and that there are dense glutamatergic projections from the ventral hippocampus to the nucleus accumbens that can influence DA and VTA neural activity (e.g., Floresco et al., 2001; Legault et al., 2000). Thus, the ventral hippocampus should also be considered as a potential locus of control for CORT-DA interactions.

Alternative approaches and future directions

Glucocorticoid receptor conditional knockdown rats

One of the advantages of a pharmacological approach, like that used in Chapter 3, is its translational potential. However, its limitation is lack of specificity. Recently, a line of glucocorticoid conditional knockdown rats was developed using CRISPR/Cas 9 technology (Scheimann et al., 2019). Specifically, these rats are genetically modified, in that they have two loxP sequences flanking exon 3 of the glucocorticoid receptor gene (Nr3c1). Upon exposure to cre-recombinase deletion of the exon is attained, and ultimately the deletion of the GR receptor, resulting in significant knockdown of GR levels (see Scheimann et al., 2019). With viral vector mediated cre one can conditionally delete the receptor within a region of interest, for example the nucleus accumbens or

prefrontral cortex, or within specific circuits, such as neurons projecting from the prefontal cortex to the nucleus accumbens. We believe this technique will be especially valuable in advancing our understanding of the role of glucocorticoids in cue-reward learning.

Mineralocorticoid receptors

While a number of reasons have been discussed throughout this dissertation to justify our focus on the Type II glucocorticoid receptor, the Type I mineralocorticoid (MR) receptor plays an important role in regulating the hypothalamic-pituitary-adrenal (HPA) axis (Herman et al., 2016). MRs are the high affinity receptor that glucocorticoids bound to under conditions of low levels (Reul & de Kloet, 1985). A balance between MR/GR in the brain is crucial in determining glucocorticoid levels (Joels & de Kloet, 1994). MRs are known to mediate certain aspects of learning (e.g., in spatial learning) (Oitzl & de Kloet, 1992), and have in fact been associated with dopamine. For example, an MR antagonist within the ventral tegmental area (VTA) attenuates the expression of a conditioned response to an aversive Pavlovian cue and results in decreased dopamine within the basolateral amygdala, a target of VTA neurons (de Oliveira et al., 2014). While the role of MR in learning has been centered around fear conditioning, its role in appetitive conditioning, and specifically incentive learning, warrants further investigation. Similarly, other components of the HPA axis (e.g. corticotropin releasing hormone) have received little attention beyond negative valence systems and should be further investigated for their potential role in reward learning.

Future directions

Immediate futures studies should assess negative valence systems of female goa-trackers and sign-trackers, and further examine the sex-dependent effects of glucocorticoids in cue-reward learning. For example, a dose-effect analysis of CORT administration should be conducted, and brain levels of both CORT and dopamine assessed concurrently in female and male rats. Further, the novel conditional GR knockdown rat line will permit cell- or region-specific manipulation of GRs in conjunction with microdialysis to identify the site of intersection between glucocorticoids and dopamine.

Concluding remarks and future directions

Work presented in this dissertation serves as evidence for a role of glucocorticoids in dopamine-dependent cue-reward learning, or incentive salience; and argues for the ongoing investigation of the glucocorticoid-dopamine integrative role. We established that differences in peripheral glucocorticoids between goal- and sign-trackers pertain to different cue-reward learning strategies, and not negative valence systems. Pharmacological manipulations demonstrated that glucocorticoids can influence the acquisition or expression of a cue-response, and that they do so in a sex- and phenotype-dependent manner. We assessed central glucocorticoids for the first time in goal- and sign-trackers; and highlighted the unique role of the dopamine system in the nucleus accumbens shell in incentive learning. The work in this dissertation is novel, as we expanded our understanding of female rat behavior and neurobiology in the context of cue-reward learning; which only a few studies have done before (Hughson et al., 2019; Phillips & Sarter, 2020; Pitchers et al., 2015). Finally, while a direct glucocorticoid-dopamine relationship was not captured, the findings reported in

this dissertation in conjunction with the existing literature support the proposed framework. That is, that glucocorticoids enhance dopamine transmission and in turn incentive cue-reward learning.

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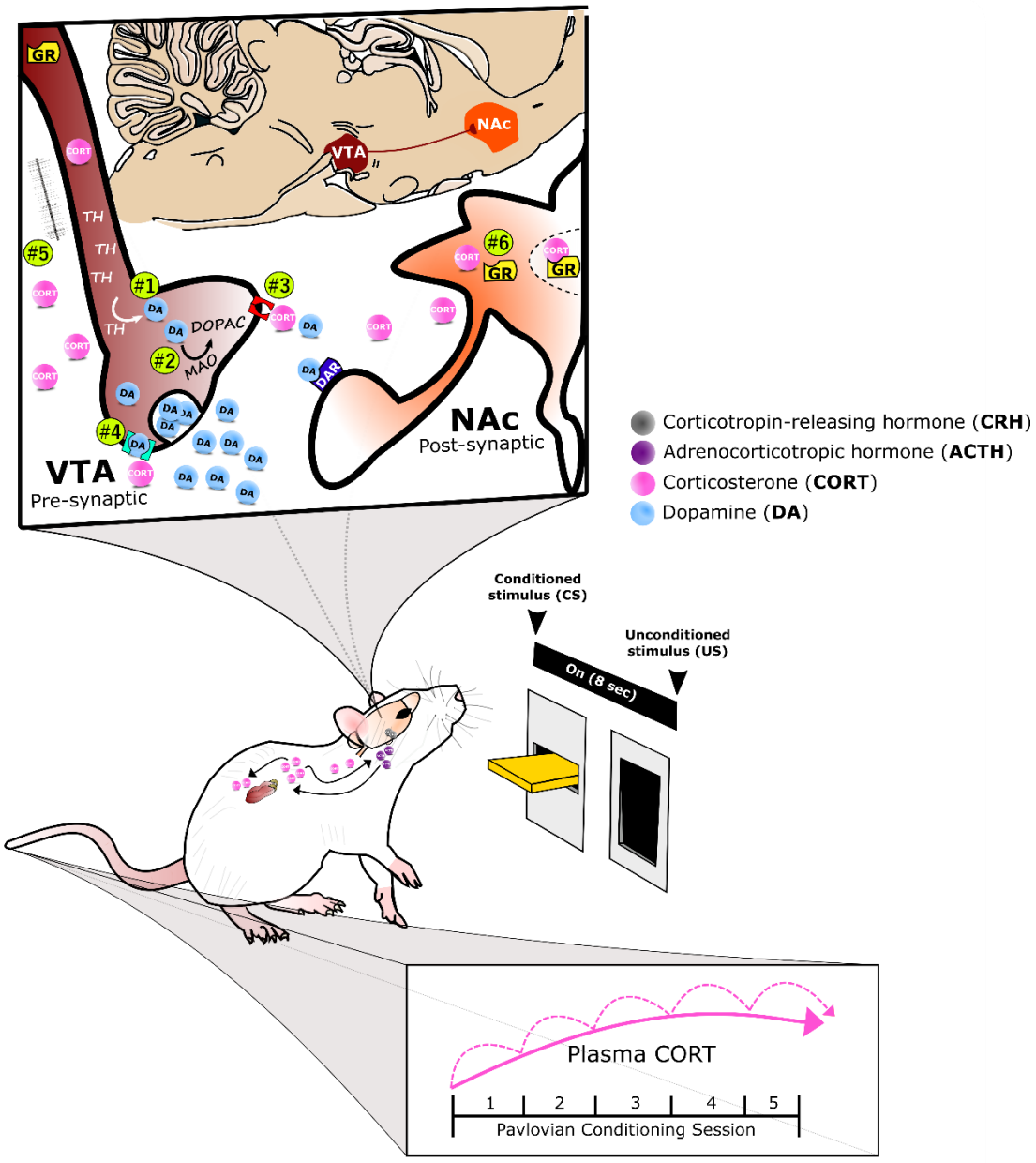

Figure 5.1 A proposed role for glucocorticoid-dopamine interactions in appetitive conditioning. The integrative response of dopamine (DA) and corticosterone (CORT) under appetitive Pavlovian conditioning is depicted. With repeated pairings of a lever-conditioned stimulus (CS) and food-unconditioned stimulus (US), CORT (pink circles) increases both peripherally and centrally, while DA (blue circles) increases within the nucleus accumbens (NAc, orange structure). Corticosterone may potentiate dopamine release by acting within the ventral tegmental area (VTA, maroon structure) to: 1) increase tyrosine hydroxylase (TH), the rate limiting enzyme of DA biosynthesis, or 2) decrease monoamine oxidase (MAO), one of the degradation enzymes of DA. Alternatively, CORT may act presynaptically to mediate DA clearance and/ or synaptic uptake by: 3) inhibiting the organic cation transporter (OCT3, red structure) or 4) the dopamine transporter. Finally, CORT may increase DA transmission 5) via glutamatergic synapses or 6) by acting directly upon glucocorticoid receptors (GR) within DA-receptive neurons.

Appendix A

Introduction

In Chapter 3, the limitations of *acquisition* studies were discussed. For example, variability and skewedness in phenotype distribution. This caveat is not present in *expression* studies, as rats are classified as goal- and sign-trackers before assessing the effect of treatment. Here we expand on the findings reported in Chapter 3 by performing “clean” expression studies. That is, classifying untreated animals as goal- or sign-trackers and then assessing the effects of CORT administration on the expression of the acquired conditioned responses. Unfortunately, due to skewed phenotype distributions, the sample size per phenotype and sex are too low to conduct meaningful analyses or draw conclusions. Thus, data are presented both across and within pehnotypes, and ongoing studies are being conducted to increase the sample sizes.

Materials and Methods

Subjects

A total of twenty-four female and twenty-four male Sprague Dawley rats from Charles River (colony R08, Raleigh, NC, USA) and from Taconic Biosciences (colony Bu016, Cambridge City, IN, USA), weighing 225-275 g upon arrival, were used. Housing conditions were identical to Chapter 3 and approved by the University of Michigan Institutional Animal Care and Use Committee.

Monitoring the estrous cycle

Female rats were monitored daily (15 :00 h) for their stage of estrous cycle by vaginal lavages. Procedures were performed identical to Chapters 3 and 4. Males were weigh daily to account for extra handling.

Behavioral testing

Pavlovian conditioned approach (PavCA) behavior

PavCA training procedures were identical to Chapter 3 with the exception of undergoing nine consecutive PavCA sessions and being characterized as goal-trackers (GTs), intermediate responders (IRs), and sign-trackers (STs) at the end of PavCA session 5 (see experimental timeline, Figure S3.1). Like Meyer et al. (2012), the averaged PavCA index from sessions 4 and 5 was used to classify phenotypes with the following cutoffs: ≤ -0.5 for a GT, ≥ 0.5 for a ST, and in between -0.5 and 0.5 for an IR, those that vacillate in between.

Conditioned reinforcement test (CRT)

For this experiment, the conditioned reinforcement test (CRT) took place twenty-four hours following the ninth, or last, PavCA session (see experimental timeline S3.1).

Pharmacological treatment

To assess the effect of systemic corticosterone (CORT) on the expression of a conditioned response rats received their first vehicle (VEH) injection immediately following the fifth PavCA session, and either VEH or CORT prior to PavCA sessions 6 through 9 (see experimental timeline, Figure S3.1).

Statistical analysis

Similar to Chapter 3, behavioral outcome measures were analyzed using the Statistical Package for the Social Sciences (SPSS) program version 26.0 (IBM, Armok,

NY, USA). Linear mixed-effects models were performed for acquisition PavCA behavior (session 1-5), using the best fit covariance structure with the lowest Akaike's information criterion for each set of data. Repeated measures analysis of variance with between subjects comparisons for Treatment and Sex were performed for averaged acquisition vs. expression PavCA Index. Univariate analysis of variance was performed for conditioned reinforcement test measures. Statistical significance was set at $p<0.05$, and Bonferroni post hoc comparisons were conducted when significant interactions were detected. One rat was excluded for not consuming the reward across multiple training sessions and an additional rat was excluded from conditioned reinforcement test due to noseport malfunction.

Results

PavCA behavior

Acquisition Phase

Lever and food-cup directed behaviors across the acquisition phase, sessions 1-5, were assessed and compared between sex and treatment group. While, treatment was not administered during this phase, Table S3.1 demonstrates that acquisition of sign- or goal-tracking behaviors was similar between sex and designated treatment groups. Phenotype differences were apparent as early as PavCA session 1 for lever-directed behaviors and session 2 for food-cup directed behaviors (see Table S3.2).

Expression phase

Independent of phenotype (i.e., collapsed), the composite PavCA index, or the propensity to sign- or goal-track in response to the lever-CS, was significantly different between the acquisition phase (averaged PavCA index from sessions 4-5) and the

expression phase (averaged PavCA index from sessions 6-9), when rats received a systemic administration of either vehicle or CORT [Effect of Phase: $F_{1,43}$=16.260, p<0.001] (Figure S3.2A-B). This difference in behavior, however, was not dependent on sex [Effect of Sex: $F_{1,43}$=3.138, p=0.084] or treatment (VEH vs. CORT) [Effect of Treatment: $F_{1,43}$=1.157, p=0.288; Phase x Treatment interaction: $F_{1,43}$=0.131, p=0.071; Phase x Sex interaction: $F_{1, 43}$=0.037, p=0.848; Phase x Treatment x Sex: $F_{1,43}$=0.017, p= 0.897].

Goal-trackers

When phenotypes were analyzed separately (based on *a priori* hypotheses), there was not a significant difference in the PavCA index between acquisition and expression for goal-trackers [Effect of Phase: $F_{1,7}$=2.454, p=0.161] (Figure S3.3A-B). Further, there was not a significant difference between sexes nor an effect of CORT on the expression of the conditioned response [Effect of Sex: $F_{1,7}$=2.327, p=0.171; Effect of Treatment: $F_{1,7}$=0.023, p=0.883; Phase x Treatment interaction: $F_{1,7}$=0.175, p=0.688; Phase x Sex interaction: $F_{1, 7}$=0.044, p=0.840; Phase x Treatment x Sex: $F_{1,7}$=0.029, p= 0.870].

Intermediate responders

For intermediate responders, there was a significant difference between the acquisition and expression phase [Effect of Phase: $F_{1,7}$=14.668, p=0.006], but again, this effect was not dependent on treatment [Effect of Treatment: $F_{1,7}$=1.553, p=0.253] or sex [Effect of Sex: $F_{1,7}$=0.252, p=0.631; Phase x Treatment interaction: $F_{1,7}$=2.656, p=0.147; Phase x Sex interaction: $F_{1, 7}$=0.321, p=0.589; Phase x Treatment x Sex: $F_{1,7}$=0.269, p= 0.620]. Overall, IRs had more positive PavCA index scores, or greater

sign-tracking, during the expression phase relative to the acquisition phase (FigureS3.3C-D).

Sign-trackers

For sign-trackers, there was not a significant difference between the acquisition and expression phase [Effect of Phase: $F_{1,21}$=3.391, *p*=0.080] for the PavCA index, nor was there an effect of sex [Effect of Sex: $F_{1,21}$=2.253, *p*=0.148] or treatment [Effect of Treatment: $F_{1,21}$=0.554, *p*=0.465; Phase x Treatment interaction: $F_{1,21}$=1.848, *p*=0.188; Phase x Sex interaction: $F_{1, 21}$=1.241, *p*=0.278; Phase x Treatment x Sex: $F_{1,21}$=0.206, *p*= 0.165] (Figure S3.3E-F).

Conditioned reinforcement test

Table S3.3 includes statistics on the behavioral measures from the conditioned reinforcement test including, nose pokes into the active vs. inactive ports (Figure S3.4A), number of lever press (Figure S3.4B), and incentive value index scores (Figure S3.4C) split by sex and treatment (Table S3.3 (top)). Overall, rats poked more readily into the active port, which resulted in the presentation of the lever. Relative to males, females, had a greater number of active nose pokes (*p*<0.001), lever presses (p=0.006), and greater incentive value index score (p=0.018). Treatment history did not influence CRT behaviors. This lack of treatment effect remained when split by phenotype (see Table S3.3 (bottom) and Figure S3.5).

Conclusion

In conclusion, while changes in behavior from the *acquisition* to *expression* phase were captured, they did not differ based on treatment, sex, or phenotype. Thus far, we have failed to replicate the effects of CORT on the expression of a conditioned

response from Chapter 3. Notably, sample size is low across groups and especially for female GTs. Ongoing studies are being conducted to increase the sample size.

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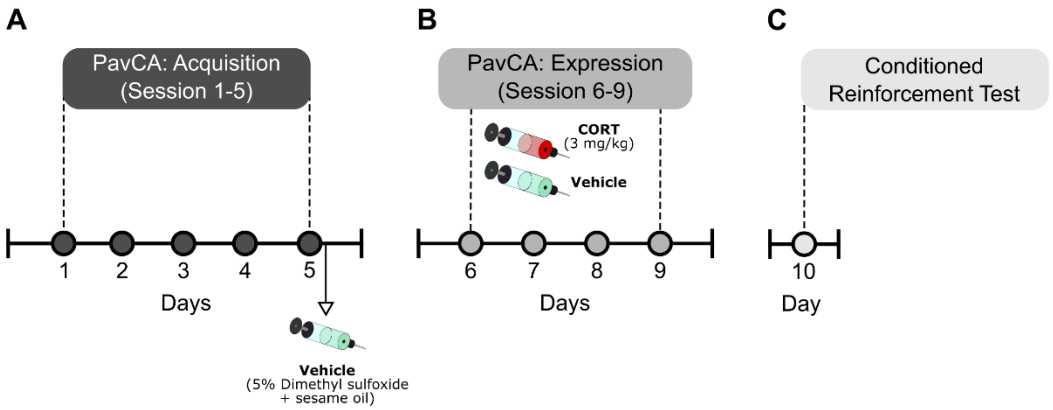
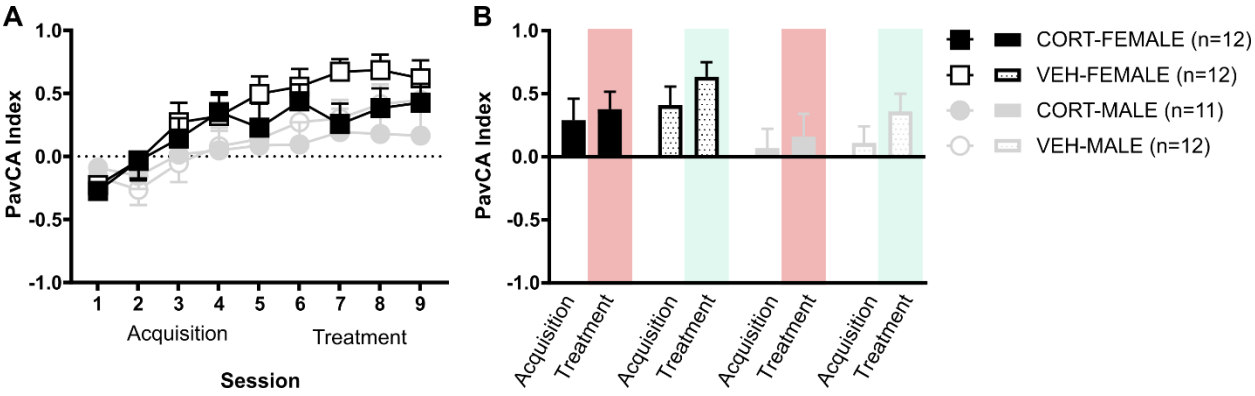
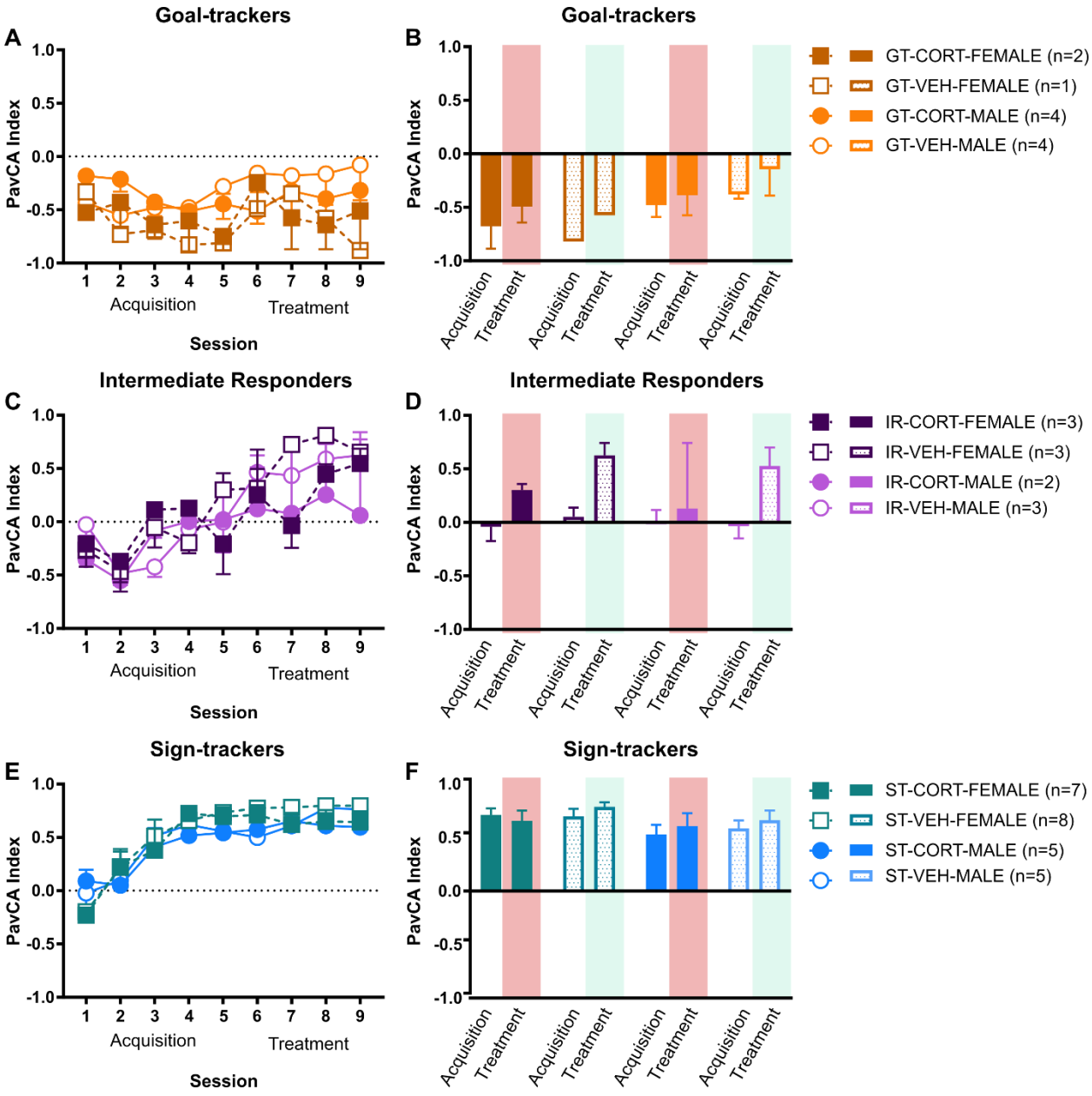


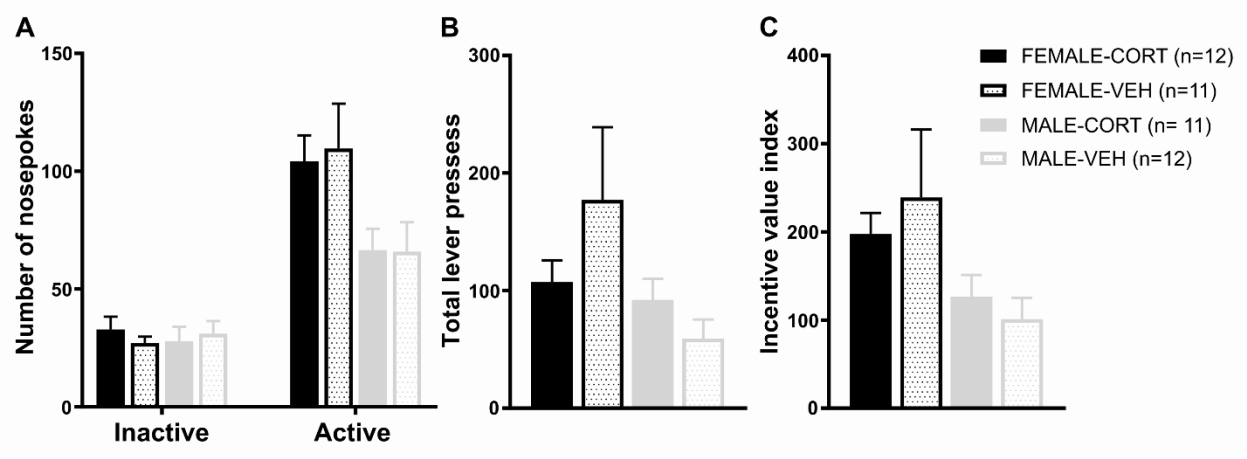
Supporting Figure S3.1 Experimental timeline. A) Rats underwent, an "acquisition phase", consisting of five consecutive Pavlovian conditioned approach (PavCA) training sessions. Following the last session, session five, all rats were systemically administered with vehicle (VEH), 5% dimethyl sulfoxide (DMSO) diluted in sesame oil. **B**) During the "expression phase", rats were administered with VEH or 3 mg/kg corticosterone (CORT) thirty minutes prior to each of four consecutive additional PavCA training sessions. **C**) On the tenth day, rats underwent a treatment-free single conditioned test (CRT).



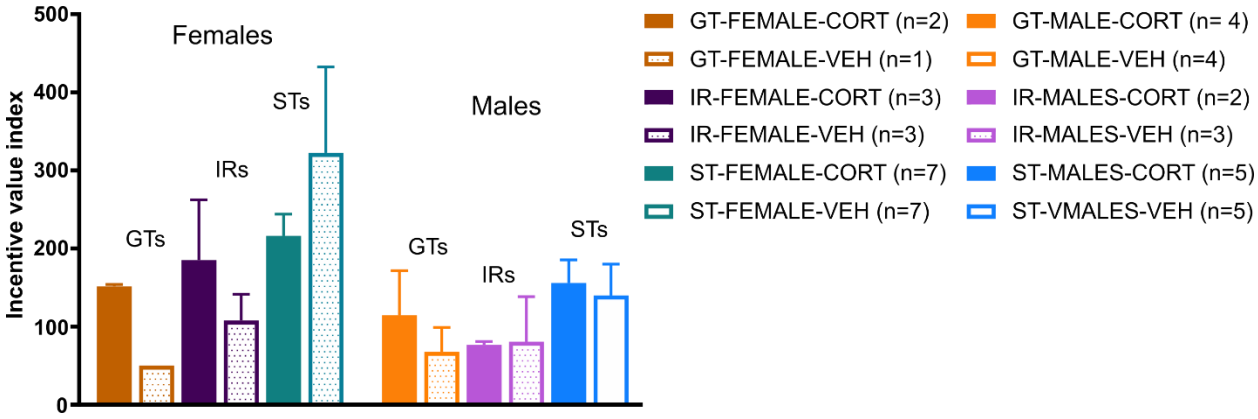
Supporting Figure S3.2 The effect of CORT on the expression of a conditioned response of female and male rats. A) Mean +/- SEM Pavlovian conditioned approach (PavCA) index across five consecutive sessions, free of treatment (i.e., acquisition phase) and four consecutive sessions (session 6-9) having received either 3 mg/kg CORT (solid shapes) or vehicle (open shapes) 30 min prior. **B**) Mean + SEM average PavCA index during the acquisition phase (averaged PAvCA index from sessions 4-5, solid bars) and the expression phase (averaged PavCA index from sessions 6-9, dotted bars). Expression phase PavCA index was significantly different from the acquisition phase, but independent of sex (black vs. gray) and treatment (solid vs. dotted).



Supporting Figure S3.3 The effect of CORT on the expression of a conditioned response of female and male goal-tracker, intermediate responder, and sign-tracker rats. A, C, E) Mean +/- SEM Pavlovian conditioned approach (PavCA) index across five consecutive sessions, free of treatment (i.e., acquisition phase) and four consecutive sessions (session 6-9) having received either vehicle or 3 mg/kg CORT. **B, D, F**) Mean + SEM average PavCA index during the acquisition phase (averaged PAvCA index from sessions 4-5, solid bars) and the expression phase (averaged PavCA index from sessions 6-9, dotted bars) for **B**) goal-trackers, **D**) intermediate responders, and **F**) sign-trackers. Expression phase PavCA index was significantly different from the acquisition phase for **D**) intermediate responders only, but independent of sex (dark vs. light purple) and treatment (solid vs. dotted).



Supporting Figure S3.4 The effect of prior CORT history on conditioned reinforcement test measures for female vs. male rats. Mean + SEM **A**) number of nosepokes into the inactive vs. active nose port, **B**) total lever contacts during the CRT, and **C**) incentive value index score [(active nosepokes + lever presses) – inactive nosepokes]. Overall rats poked more readily into the active port ($p<0.001$), but females did so to a greater extent ($p<0.001$), as well as had more lever presses ($p=0.006$) and greater incentive value index score ($p=0.018$), relative to males. Treatment history had no effect.



Supporting Figure S3.5 The effect of prior CORT history on conditioned reinforcement test for female and male goal-tracker, intermediate responder, and sign-tracker rats. Mean + SEM incentive value index scores. The extent to which the lever-CS served as a reinforcer did not depend on sex, phenotype, or treatment history.

Acquisition PavCA Behavior

Females vs. Males	Lever Contacts			Lever Contact Probability			Lever Contact Latency		
Sign-tracking	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Session	4, 51.41	7.465	p<0.001	4, 85.51	17.853	p<0.001	4, 77.78	14.168	p<0.001
Effect of Sex	1,34.5	0.702	p=0.408	1, 37.46	0.132	p=0.718	1, 37.23	1.050	p=0.312
Effect of Phenotype	2, 34.5	20.693	p<0.001	2, 37.46	35.4761	p<0.001	2, 37.23	28.793	p<0.001
Effect of Treatment	1, 34.5	0.037	p=0.849	1, 37.46	0.615	p=0.438	1, 37.23	0.284	p=0.597
Session*Sex	4, 51.41	2.129	p=0.90	4, 85.51	1.674	p=0.164	4, 77.78	0.1.782	p=0.141
Session*Phenotype	8, 51.41	4.67	p<0.001	8, 85.51	4.625	p<0.001	8, 77.78	0.6.172	p<0.001
Session*Treatment	4, 51.51	0.742	p=0.568	4, 85.512	1.749	p=0.147	4, 77.78	1.541	p=0.199
Session*Sex* Phenotype	10, 49.7	1.492	p=0.171	10, 67.51	1.405	p=0.197	10, 69.07	1.199	p=0.307
Session*Sex* Treatment	5, 49.7	0.180	p=0.969	5, 67.51	1.634	p=0.163	5, 69.074	0.627	p=0.679
Session*Phenotype* Treatment	10, 49.7	0.606	p=0.801	10, 67.51	0.681	p=0.739	10, 69.07	0.754	p=0.671
Session*Sex* Phenotype* Treatment	10, 49.7	0.266	p=0.986	10, 67.51	0.875	p=0.561	10, 69.07	0.306	p=0.977

	Food-cup Entries			Food-cup Entry Probability			Food-cup Entry Latency		
Goal-tracking	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Session	4, 81.93	3.957	p=0.005	4, 80.85	5.666	p<0.001	4, 49.02	7.434	p<0.001
Effect of Sex	1, 34.98	0.332	p=0.568	1, 35.0	1.588	p=0.216	1, 38.34	3.763	p=0.060
Effect of Phenotype	2, 34.98	14.989	p<0.001	2, 35.0	13.735	p<0.001	2, 38.34	23.490	p<0.001
Effect of Treatment	1, 34,98	0.057	p=0.813	1, 35.0	0.374	p=0.545	1, 38.34	0.062	p=0.805
Session*Sex	4, 81.93	0.272	p=0.895	4, 80.85	1.564	p=0.192	4, 49.02	0.622	p=0.649
Session*Phenotype	8, 81.93	10.151	p<0.001	8, 80.85	9.301	p<0.001	8, 49.02	11.343	p<0.001
Session*Treatment	4, 81.93	0.128	p=0.972	4, 80.85	0.366	p=0.832	4, 49.02	0.288	p=0.885
Session*Sex* Phenotype	10, 86.3	1.749	p=0.083	10, 86.3	1.068	p=0.396	10, 49.48	2.323	p=0.025
Session*Sex* Treatment	5, 86.3	1.255	p=0.291	5, 86.3	1.134	p=0.349	5, 49.48	1.174	p=0.335
Session*Phenotype* Treatment	10, 86.3	1.188	p=0.310	10, 86.3	0.708	p=0.715	10, 49.48	0.600	p=0.806
Session*Sex* Phenotype* Treatment	10, 86.3	1.197	p=0.304	10, 86.3	0.974	p=0.472	10, 49.48	1.679	p=0.112

Supporting Table S3.1 Linear mixed model analysis for sign- and goal-tracking behaviors during the "acquisition phase". Effect of Session, Sex, Phenotype, Treatment, and Session x Sex, Session x Phenotype, Session x Treatment, Session x Sex x Phenotype, Session x Sex x Treatment, Session x Phenotype x Treatment, and Session x Sex x Phenotype x Treatment interactions were analyzed.

Phenotype comparisons

	Sign-tracking Lever Contacts					**Goal-tracking** Food cup Entries				
	1	2	3	4	5	1	2	3	4	5
GTs vs. IRs	p=0.661	p=1.00	p=1.00	p=0.801	p=0.305	p=0.840	p=1.00	p=0..300	p=0.010*	p<0.001*
GTs vs. STs	p=0.004*	p=0.028*	p<0.001*	p<0.001*	p<0.001*	p=0.258	p=0.009*	p=0.020*	p<0.001*	p<0.001*
STs vs. IRs	p=0.097	p=0.018*	p=0.001*	p<0.001*	p=0.005*	p=1.00	p<0.001*	p=0.905	p=0.054	p=0.012*

	Lever Contact Probability					Food cup Entry Probability				
	1	2	3	4	5	1	2	3	4	5
GTs vs. IRs	p=0.202	p=1.00	p=0.063	p=0.008*	p=0.025*	p=1.00	p=1.00	p=0.676	p=0.031*	p=0.030*
GTs vs. STs	p<0.001*	p=0.004*	p<0.001*	p<0.001*	p<0.001*	p=0.350	p=0.041*	p=0.017*	p<0.001*	p<0.001*
STs vs. IRs	p=0.090	p=0.002*	p<0.001*	p<0.001*	p=0.016*	p=0.899	p=0.002*	p=0.301	p=0.014*	p=0.001*

	Lever Contact Latency					Food cup Entry Latency				
	1	2	3	4	5	1	2	3	4	5
GTs vs. IRs	p=0.590	p=1.00	p=0.338	p=0.183	p=0.198	p=1.00	p=1.00	p=0.586	p<0.001*	p=0.001*
GTs vs. STs	p=0.002*	p=0.008*	p<0.001*	p<0.001*	p<0.001*	p=1.00	p=0.006*	p=0.024*	p<0.001*	p<0.001*
STs vs. IRs	p=0.057	p=0.007*	p<0.001*	p<0.001*	p=0.003*	p=1.00	p=0.001*	p=0.479	p=0.030*	p=0.001*

Supporting Table S3.2 Phenotype comparisons. Bonferroni post hoc comparisons between Phenotype for each PavCA session are reported. Sign-tracking and goal-tracking behaviors are included for male and female rats combined.

Conditioned Reinforcement Test

Females vs. Males	Nosepokes			Lever Contacts			Incentive value index		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 84	0.006	p=0.939	1, 42	0.016	p=0.900	1, 42	0.035	p=0.852
Effect of Noseport	1, 84	63.958	p<0.001						
Effect of Sex	1, 84	8.408	p=0.005	1, 42	3.572	p=0.006	1, 42	6.069	p=0.018
Treatment*Noseport	1, 84	0.066	p=0.798						
Treatment*Sex	1, 84	0.010	p=0.919	1, 42	0.568	p=0.455	1, 42	0.617	p=0.437
Sex*Noseport	1, 84	7.963	p=0.006						
Sex*Noseport* Treatment	1, 84	0.289	p=0.593						

Females vs. Males by Phenotype	Nosepokes			Lever Contacts			Incentive value index		
	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value	df_1, df_2	F-Value	P-Value
Effect of Treatment	1, 68	0.009	p=0.925	1, 34	0.260	p=0.613	1, 34	0.199	p=0.658
Effect of Noseport	1, 68	35.451	p<0.001						
Effect of Sex	1, 68	2.17	p=0.145	1, 34	0.698	p=0.409	1, 34	1.827	p=0.185
Effect of Phenotype	2, 68	1.721	p=0.187	2, 34	2.233	p=0.123	2, 34	2.692	p=0.082
Treatment*Noseport	1, 68	0.008	p=0.929						
Treatment*Sex	1, 68	0.003	p=0.856	1, 34	0.004	p=0.951	1, 34	0.002	p=0.962
Treatment* Phenotype	2, 68	0.032	p=0.968	2, 34	0.551	p=0.581	2, 34	0.638	p=0.534
Sex*Noseport	1, 68	3.753	p=0.057						
Phenotype* Noseport	2, 68	1.649	p=0.200						
Sex*Phenotype	2, 68	1.294	p=0.281	2, 34	0.507	p=0.607	2, 34	0.480	p=0.623
Sex*Noseport* Treatment	1, 68	0.000	p=0.996						
Sex*Phenotype* Treatment	2, 68	0.011	p=0.989	2, 34	0.598	p=0.556	2, 34	0.580	p=0.565
Noseport* Phenotype*Treatment	2, 68	0.349	p=0.707						
Noseport* Sex*Phenotype	2, 68	0.43	p=0.867						
Noseport* Sex*Phenotype*Treatment	2, 68	0.180	p=0.836						

Supporting Table S3.3 Univariate analysis of variance for conditioned reinforcement test (CRT). Nose pokes (left), total lever contacts (middle), and incentive value index (right) were assessed. Effect of Treatment, Noseport, Sex, and interactions were analyzed for female and male rats (top). Effect of Treatment, Noseport, Sex, Phenotype, and interactions were analyzed for females and males split by Phenotype (bottom).

Appendix B

Introduction

The microdialysis methods described in Chapter 4 were initiated by lowering the microdialysis probe into the surgically implanted guide cannula to reach the nucleus accumbens shell (NAcS). Prior to dialysate collections, a 2-hr acclimation period was implemented, with artificial cerebral spinal fluid (aCSF) continuously perfusing at a rate of 1.3 μl/ min. The amount of time allowed between probe insertion and dialysate collection is thought to be critical in capturing stable and functional extracellular dopamine (DA) levels (for review, see Robinson & Camp, 1991). Local disruptions triggered by lowering the probe (e.g., blood flow) have been reported to stabilize after several hours (Benveniste et al., 1987), and thus, are minimized by allowing an overnight acclimation period. Throughout the years, probe optimization, including shape (Westerink & De Vries, 1988) and size (Benveniste & Huttemeier, 1990), have decreased damage-induced nonspecific neurotransmitter release. While in our own hands a 2-hr acclimation period has successfully captured DA changes within the NAcS (Campus et al., 2019), seminal studies have established tetrodotoxin (TTX)- or Ca^{2+}-sensitivity as an indicator of "functional" DA release (e.g.,Westerink et al., 1988). Thus, the following study assessed Ca^{2+}-sensitivity following a 2-hr vs. 18-hr (i.e., overnight) acclimation period using the same microdialysis probes as those described in Chapter 4 (2 mm length, .5 mm outer diameter, CMA 12; Harvard Apparatus, Holliston, MA).

Materials and Methods

Subjects

A total of six female and six male Sprague Dawley rats, weighing between 225-275 g arrived from Taconic Biosciences (colony BU016, Cambridge City, IN, USA). Upon arrival, rats were paired-housed with the same sex in standard acrylic homecages in a temperature controlled room (22 ± 2°C). A 12 hr light: dark cycle (lights on at 06:00 or 07:00 depending on daylight savings time) and *ad libitum* access to food and water were instituted for the duration of the study. Rats remained undisturbed and were allowed to acclimate for four days prior to surgery. Following surgery, rats were single-housed and allowed seven days for recovery. All experimental procedures took place during the light cycle (between 10:00 to 17:00 h) and were approved by the University of Michigan Institutional Animal Care and Use Committee.

Surgical procedures

As described in Chapter 4, rats underwent surgery for unilateral guide-cannula implantation targeting the nucleus accumbens shell (+1.7 mm AP; ±0.8 mm ML; -0.6 mm DV from bregma), with the left and right hemisphere counterbalanced.

Microdialysis

Sampling

Extracellular levels of dopamine (DA) within the nucleus accumbens shell (NAcS) were assessed under baseline conditions during which rats were freely moving inside their standard acrylic homecage enclosed by a sound attenuating chamber. Rats were assigned to undergo either a 2- (n=5) or 18-hr (n=4) acclimation period following probe insertion. The 18-hr group was tethered overnight with artificial cerebral spinal fluid (aCSF) perfusing at a rate of 0.5 μl/ min. The composition of this “normal” aCSF was

identical to that of Chapter 4: 145 mM NaCl, 2.68 mM KCl, 1.40 $CaCl_2$, 1.01 mM $MgSO_4$, 1.55 mM Na_2HPO_4 (dibasic), 0.45 mM NaH_2PO_4 (monobasic), and 0.25 mM ascorbic acid diluted to 1:1000. On the day of collection, the perfusion rate was adjusted to 1.3 μl/ min and an additional 2-hr acclimation period was implemented at this new rate for rats left overnight. At this time (~10:00 h), microdialysis probes were inserted for rats belonging to the 2-hr group, and normal aCSF was perfused at a 1.3 μl/ min for 2 hrs. Once the acclimation period concluded for all rats, dialysate samples were collected every 10 min (to mimic methods from Chapter 4) for 60 min. To assess DA levels under "Ca^{2+}-free" conditions, normal aCSF was replaced with a Ca^{2+} free and high magnesium aCSF perfusate (similar to, Westerink et al., 1988): 145 mM NaCl, 2.68 mM KCl, **2.41mM $MgSO_4$**, 1.55 mM Na_2HPO_4 (dibasic), 0.45 mM NaH_2PO_4 (monobasic), and 0.25 mM ascorbic acid diluted to 1:1000. Before resuming collection, the "Ca^{2+} free" aCSF was allowed to flow for 30 min. Based on Westerink et al. (1988), who observed more than a fifty percent decrease in DA after 60 min, an additional 90 min of collection took place under these conditions.

Benzoyl chloride derivatization

Derivatization was performed identical to Chapter 4. Each sample consisted of a 13 μl volume, only 3 μl were used for derivatization and then stored at -80 °C until they were analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS).

HPLC-MS

HPLC-MS methods were performed by the Kennedy lab as described in Chapter 4.

Histology

To identify probe placement within the NAcS, brain slides (2.76-0.96 mm from bregma) were mounted on glass slides, stained with Cresyl-violet (Sigma-Aldrich, St. Louis, MO), and covered-slipped. A Leica DM 1000 light microscope was used to verify placement, and only rats with placement within the NAcS were added to the analysis.

Statistical analysis

Dopamine levels were analyzed using the Statistical Package for the Social Sciences (SPSS) program version 26.0 (IBM, Armok, NY, USA). Repeated measures analysis of variance with a between subjects comparison of acclimation period (2- vs. 18-hr) was performed to compare dopamine under normal vs. Ca^{2+} free aCS, while a univariate analysis of variance was performed for percent change dopamine levels. Statistical significance was set at $p<0.05$, and Bonferroni post hoc comparisons were conducted when significant interactions were detected. A total of five rats were not included in the analysis for the following reasons: (n=1) euthanized following surgery, (n=1) euthanized due to head cap detachment, (n=1) unsuccessful collections, (n=1) poor probe placement, (n=1) detected as an outlier by SPSS. All figures were made using GraphPad Prism 8.

Results

Dopamine

Overall, dopamine levels were significantly different when undergoing a 2- vs. 18-hr acclimation period [Effect of Group: $F_{1,5}=8.574$, $p=0.033$], with those in the 18-hr group (n=3, $\bar{x}=0.64$ nM) showing lower levels of DA, relative to the 2-hr group (n=4, $\bar{x}=1.72$ nM). However, Ca^{2+}-sensitivity was not significantly different between groups

[Effect of aCSF: $F_{1,5}$=13.586, p=0.014; aCSF x Group interaction: $F_{1,5}$=2.965, p=0.146]. DA levels decreased with the removal of Ca^{2+} and increase of magnesium in the perfusing aCSF (Figure S4.1A). Additionally, the percent change from "normal" to "Ca^{2+}-free" aCSF was not significantly different between the 2-hr ($\bar{x}$= -53.4%) vs. 18-hr group ($\bar{x}$= -48.6%) [Effect of Group: $F_{1,5}$=2.51, p=0.637] (Figure S4.1B). Thus, while DA levels did differ when rats were acclimated for 2- vs. 18-hrs following microdialysis probe insertion, the sensitivity to Ca^{2+} was similar, allowing us to conclude that the 2-hr acclimation period likely did not affect the results reported in Chapter 4.

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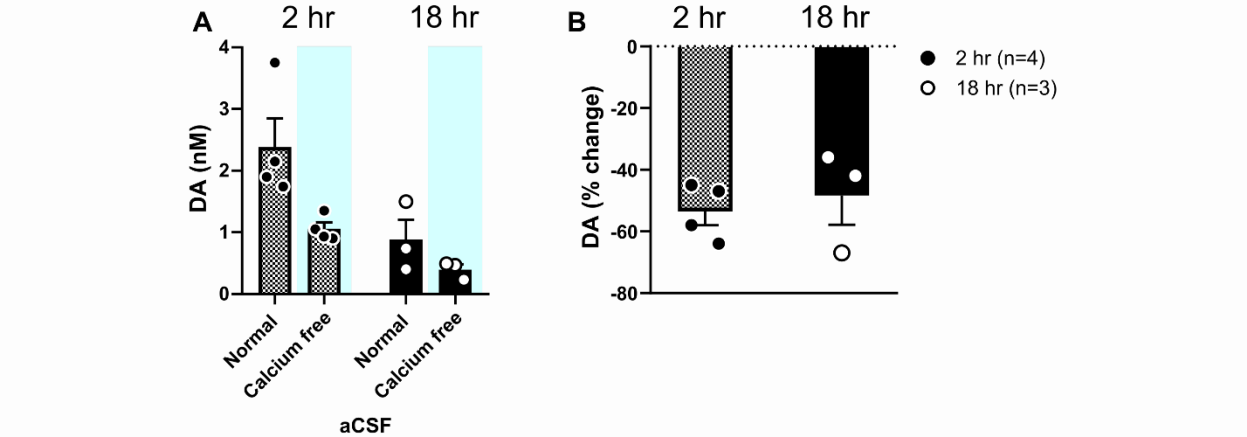

Supporting Figure S4.1. Ca^{2+}-sensitivity following a 2-hr vs. 18-hr acclimation period. Mean +/- SEM **A**) dopamine (DA) extracellular levels within the nucleus accumbens shell of male (2-hr (n=3), 18-hr (n=2)) and female (2-hr (n=1), 18-hr(n=1)) rats following a 2- vs 18-hr acclimation period under "normal" (averaged from 60 min) vs. "Ca^{2+}-free" and high magnesium (averaged from 90 min) aCSF perfusate conditions. **B**) percent change DA when switching from normal to Ca^{2+}-free aCSF. **A**) Overall, an 18-hr acclimation period resulted in lower DA levels, relative to a 2-hr period (p=0.033). **A-B**) However, Ca^{2+}-sensitivity was similar between groups.