NEUROCOGNITIVE INVESTIGATIONS OF HABITUAL BEHAVIOR MODIFICATION

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ABSTRACT

Theresa Helen McKim: Neurocognitive Investigations of Habitual Behavior Modification
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Addiction is a disorder characterized by maladaptive associative learning processes in which behavior can result despite negative health outcomes. Research from human and animal models suggests that dysfunction within frontostriatal neural circuitry may contribute to a shift from goal-directed to habit-based action selection. The goal of the present dissertation was to examine the impact of acute psychosocial stress and non-invasive transcranial alternating current stimulation on increasing and reducing habitual responding, respectively. We assessed the importance of stress timing on potentiating habitual responding in healthy males in Chapter 2 and found that stress prior to execution and learning of S-R associations increased perseverative errors. The underlying biological mechanism of this shift in behavior related to sympathetic activation; we found that males that were able to mount a parasympathetic response to counteract the biological effects of stress were less likely to perseverate. Similarly, Chapter 3 was designed to examine the relationship between stress timing and menstrual cycle phase effects on habitual responding in healthy females. In contrast to our male results, we showed that regardless of menstrual cycle phase (menstrual versus luteal) and stress timing, females did not show increased perseverative responding. These results demonstrated differences in the experience and biological response to acute psychosocial stress, and suggested that differences in ovarian hormone levels may contribute to behavior under conditions of stress. In Chapter 4 we tested the use of non-invasive transcranial alternating current stimulation in healthy controls and individuals with an

addiction history to diminish perseverative errors after response devaluation. Contrary to our predictions, true versus sham stimulation increased perseverative errors in healthy controls, while there were more subtle improvements in performance in the addiction history group, not specific to perseverative responding. Together, these data demonstrate conditions in which goal-directed behavior can be shifted toward habit-based actions, and suggest that concomitant shifts from top-down (prefrontal) to bottom-up (striatal) control within the brain contributes to changes in these response selection strategies. More broadly, these findings implicate frontostriatal circuitry and habitual behaviors as a highly promising research area to develop novel treatment methods for disorders characterized by intractable behaviors.

In loving memory of my sister, Anne Elizabeth McKim.

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LIST OF ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AUDIT	Alcohol use disorders test
BART	Balloon analog risk task
BIS	Barratt Impulsivity Scale
BOLD	Blood oxygen level dependent
BPM	Beats per minute
BSMSS	Barratt simplified measure of social status
CAARS	Connors' adult ADHD rating scale
CPT	Cold pressor task
DLPFC	Dorsolateral prefrontal cortex
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
DSM-V	Diagnostic and statistical manual
DTI	Diffusion tensor imaging

DUSI	Drug use screening inventory
EEG	Electroencephalography
FAM	Familiar set
fMRI	Functional magnetic resonance imaging
FTQ	Family tree questionnaire
HABIT	Hidden association between images task
HF	High frequency
HPA	Hypothalamic pituitary adrenal
HRV	Heart rate variability
Hz	Hertz
IBI	Interbeat interval
IGT	Iowa gambling task
IQ	Intelligence quotient
lnRMSSD	Natural logarithm transformed RMSSD
LOC	Locus of control scale
MIST	Montreal imaging stress test
mPFC	Medial prefrontal cortex

NOV	Novel set
OCD	Obsessive compulsive disorder
OFC	Orbitofrontal cortex
OSPAN	Operation span
PFC	Prefrontal cortex
PMC	Premotor cortex
RMSSD	Root mean square of successive differences
RT	Reaction time
SAS	Statistical and computing software
SECPT	Socially evaluation cold pressor test
SES	Socioeconomic status
SILS	Shipley institute of living scale
SNS	Sympathetic nervous system
SPSS	Statistical package for the social sciences
S-R	Stimulus-response
STAI	State-Trait anxiety inventory
SUD	Substance use disorder

tACs	Transcranial alternating current stimulation
TAF	Thought action fusion scale
tDCs	Transcranial direct current stimulation
TLT	Tower of London task
TMS	Transcranial magnetic stimulation
TSST	Trier social stress test
UNC	University of North Carolina
VBM	Voxel based morphometry
vmPFC	Ventromedial prefrontal cortex
WCST	Wisconsin card sorting t

CHAPTER 1

GENERAL INTRODUCTION

Addiction is a chronic disorder characterized by cycles of repeated drug or alcohol use, withdrawal, abstinence periods and relapse, which reinitiates the drug or alcohol use. Due to the cyclic nature of the disorder, it poses a large burden on society and necessitates access to treatment resources. In 2015, the National Survey on Drug Use and Health estimated that 21.7 million individuals over the age of 12 needed treatment for a drug or alcohol problem (SAMSHA, 2016). In contrast, only 10.8% (2.3 million) of those people needing treatment were able to receive it at a treatment facility. When considering alcohol use problems alone, it is the fourth leading cause of preventable death in the United States (Stahre et al., 2014). These statistics suggest that there are large economic, social/familial, and health costs to both the individual as well as society in terms of lost productivity and health care. Given the high prevalence of these disorders as well as high relapse rates, there is an increasing need for effective treatment options and research to investigate the underlying neurobiological mechanisms of dysfunction.

Our previous research demonstrated that individuals with a substance use disorder (SUD) history transition to habit-based response strategies more rapidly than healthy controls (McKim et al., 2016a). Actions that are repeated and practiced over time transition to habits, which are evoked by stimuli and occur regardless of the associated outcome value of the initiated behavior (Dickinson, 1985). In daily life, habitual responses to stimuli within our environment allow us to

perform daily routines that are automatized and efficient, affording the ability to simultaneously use cognitive resources for more demanding tasks, such as creating a plan for future action selection. While these automatized behaviors are advantageous in many situations, it is possible for such behaviors to become detrimental to one's health if they become inflexible. For example, we colloquially, refer to these behaviors as 'bad habits'. In the case of alcohol use, it may occur despite serious adverse health consequences such as cirrhosis of the liver. This sort of disregard for adverse outcomes of substance use is a diagnostic criterion for addiction, suggesting that addiction can be construed, in some cases, as a disorder of maladaptive habitual actions.

In addition to routine behaviors, cognitive flexibility and goal-directed action selection allow us to make novel decisions and employ forward planning strategies when in an unfamiliar context. These cognitive functions have been ascribed to the prefrontal cortex (PFC), whereas habitual responding is regulated by the striatum (Balleine and Dickinson, 1998; Balleine and O'Doherty, 2010; Dolan and Dayan, 2013). Whether these findings from healthy individuals also apply to the neurobiological mechanism underlying the shift in behavior from goal-directed to habitual in the context of addiction that we previously demonstrated is currently under investigation. Evidence from human studies of addiction implicate deficits in frontostriatal circuitry, which may contribute to an overreliance on habit-based actions. Additionally, stress has been shown to shift the balance of action selection toward the use of habit-based choices, and this may be an underlying mechanism that facilitates stress-induced relapse in addiction. To further probe these hypotheses, we will use two approaches to modify behavior: stress and non-invasive brain stimulation. We will use psychosocial stress to promote habit-based responding and test manipulation of stress timing to disentangle effects on acquisition versus overtraining in healthy controls. We will further probe our behavioral findings in SUDs to investigate the underlying

neurobiological mechanisms by utilizing transcranial alternating current stimulation (tACs) of the PFC to prevent the shift toward habitual behavior in individuals with an SUD history. To substantiate our proposed studies and rationale for choice of methods and target brain regions, we will discuss the translational findings from animal models of habits and addiction, and review their neural correlates. We will then discuss supporting evidence for the biological actions of stress within frontostriatal circuitry and the proposed mechanisms of action for non-invasive stimulation of PFC to promote top-down control over habitual responding.

Animal Models of Goal-Directed and Habit Behavior

Goal-directed and habitual responding have been investigated extensively in animals, resulting in established approaches for distinguishing these two classes of actions. In general, these studies employ canonical outcome manipulation tests after an animal has been trained to perform an operant response to receive a reward. Initially, the animal is trained that one action (e.g. right lever press) results in the delivery of one reward (e.g. a chocolate pellet), while an alternative action (e.g. left lever press) results in delivery of an alternative reward (e.g. a sucrose pellet). Once training has been established, the value of one of the actions is manipulated via either degrading the action-outcome contingency ("contingency degradation"), or by devaluing the outcome of that action via sensory specific satiety or conditioned taste aversion, while the value of the other action and/or reward remains intact. In contingency degradation, the probability of the reward being delivered changes, such that the reward may be given non-contingently with lever press behavior. Goal-directed behavior is defined as when an animal continues to take actions with intact reinforcement probability, but withholds actions associated with the degraded outcome, in which a reward is delivered regardless of actions. In contrast, habitual behavior is evident if the animal

continues to press the lever with the degraded outcome and does not register the change in probability of reward delivery, and thus the need to alter behavioral responding. An alternative to contingency degradation is the use of sensory specific satiety and/or conditioned taste aversion to manipulate outcome value. The motivation for behavioral responding is manipulated in sensory specific satiety by providing ad libitum access to one reward type prior to testing responses. Conditioned taste aversion pairs one reward type with a compound such as lithium chloride to induce sickness. Once the devaluation manipulation has occurred (after training), the animal's responding is then tested to determine whether their actions are guided by the value of the rewards or whether their actions continue to occur regardless of the current outcome value. If the animal continues to take an action after the outcome of that action has been devalued, that action is classified as habitual, due to a failure to update the current value of the reward; alternatively, if the corresponding action diminishes or ceases, this is taken as evidence that the action is still guided by motivation to obtain the outcome of that action, and is performed in a goal-directed manner.

Research into the neural basis of behavioral control of action selection in animal models has demonstrated that frontostriatal circuitry is necessary for both goal-directed and habitual responses (see McKim et al., 2016b for recent review). Specifically, the prelimbic portion of the medial prefrontal cortex (mPFC) is necessary for the acquisition of goal-directed behavior (Killcross and Coutureau, 2003), and the dorsomedial striatum (DMS), the rodent homolog of the caudate, is also important for sensitivity to outcome devaluation and contingency degradation procedures (Yin et al., 2005a; Yin et al., 2005c), which underlies goal-directed behavior. In contrast, the infralimbic mPFC and the dorsolateral striatum (DLS), the rodent putamen homolog, have been shown to be critical for habit formation; inactivation of these regions reinstates sensitivity to outcome devaluation and produces goal-directed responding (Coutureau and

Killcross, 2003; Killcross and Coutureau, 2003; Yin et al., 2004, 2006b; Stalnaker et al., 2010). Recent work has also demonstrated that the rodent mPFC, the putative rodent homolog of the dorsolateral PFC (DLPFC) (Farovik et al., 2008), flexibly controls action selection by facilitating switching between well-practiced and newly acquired T-maze running behavior (Smith et al., 2012b; Smith and Graybiel, 2013). This experimental evidence from animal models suggests that learning results in changes within frontostriatal circuits that ultimately converge within the striatum, which acts as a hub to integrate incoming signals from various neural regions to control behavior.

Paradigms to Measure Habitual responding in Humans

Human studies of instrumental learning to investigate neural control of goal-directed and habitual actions were initially modeled directly on experimental paradigms developed for use in rodents. For example, humans have been trained to acquire food rewards that are then devalued through consumption to satiety. This approach has been used to demonstrate that reduced responding during extinction testing, evidence of a goal-directed response strategy, is associated with decreased orbitofrontal cortex (OFC) activity for devalued responses (Valentin et al., 2007). In contrast, more extended instrumental training renders responses insensitive to outcome devaluation through satiety, with corresponding neuroimaging data revealing increased activation in the posterior putamen/globus pallidus relative to initial response selection learning (Tricomi et al., 2009). Other neuroimaging research in human subjects has implicated a distributed set of brain regions within the corticostriatal network in goal-directed behavior, including encoding of outcome value representations in the ventromedial PFC (vmPFC), and action-outcome contingency encoding in the anterior caudate nucleus, which parallels evidence from animal

studies of the DMS (Tricomi et al., 2004; Valentin et al., 2007; Tanaka et al., 2008; de Wit et al., 2009b; Mattfeld et al., 2011; O'Doherty, 2011).

A second commonly used task to investigate habit in humans utilizes an incongruent task condition to induce response conflict during action selection; overlapping associations between stimuli and outcome images are present in incongruent trials to preferentially engage a habitual response strategy, as using a goal-directed strategy is disadvantageous (de Wit and Dickinson, 2009). In contrast, congruent trials can be successfully learned and performed by either goal-directed or habit-based response selection. Neuroimaging data from this task demonstrate that goal-directed action selection is accompanied by increased vmPFC activity. Additionally, several task stimuli were devalued to result in a loss of points, and participants were tested for 'slips-of-action' or continued responding for the now devalued images, similar to a 'go-no-go' paradigm. Diffusion tensor imaging (DTI) analysis of this aspect of the task showed that white matter connectivity between the vmPFC and caudate positively correlated with goal-directed choice behavior, while connectivity strength between the anterior putamen and the premotor cortex (PMC) negatively correlated with responses dependent upon outcome value, taken as evidence of habit-based actions (de Wit et al., 2012). Further analysis of putamen structure using voxel-based morphometry (VBM) also showed that individuals with greater gray matter density in the putamen were more likely to show vulnerability to slips-of-action during testing. These data from healthy controls provide further support for the neural basis of goal-directed versus habitual control within corticostriatal neural circuitry.

While the aforementioned tasks used in human subjects may involve extended training (across days) or multiple task epochs, including training and test, an alternative, computationally-based paradigm has been developed to examine the parallel engagement of goal-directed and

habitual choice within a reinforcement learning framework (Daw et al., 2005). According to reinforcement learning theory, action selection is either under control of the outcome and helps to predict long-term gains to update an internal model of future behavior (model-based), or is based on learned responses to stimuli preceding rewards and ultimately to a cached outcome value (model-free). To measure the contributions of these two systems to choice behavior, a two-step decision task is commonly employed in which model-free (or habitual) choices are repeated based on previous experience with rewarded trials, whereas model-based (or goal-directed) actions plan future reward choices based on constantly updating outcome experience to optimize reward accumulation. An initial functional magnetic resonance imaging (fMRI) study with this task demonstrated a reward prediction error signal related to model-free control in the ventral striatum, as well as a state prediction error signal related to model-based control in the lateral PFC and posterior inferior parietal cortex (Glascher et al., 2010). More recent imaging studies with this task have further demonstrated that model-based or model-free response selection representation in the striatum is integrated by prefrontal brain regions, such as the vmPFC, inferior lateral prefrontal, or frontopolar cortex (Wunderlich et al., 2012; Lee et al., 2014). Action selection in this task can be accounted for by either type of action selection (goal-directed or habit-based), which therefore affords the opportunity to study individual differences in behavioral response strategy. However, there is current debate over whether model-based and model-free strategies are separate, or whether model-free computations are actually a subprocess of model-based behavior and thus goal-directed (Miller et al., 2016). To date, this task has been used in combination with transcranial magnetic stimulation (TMS) of the DLPFC and transcranial direct current stimulation (tDCs) of DLPFC brain areas, with results showing that TMS can shift behavior toward a model-free strategy (Smittenaar et al., 2013), but tDCS of the DLPFC did not alter action selection strategy in healthy

controls (Smittenaar et al., 2014). These inconsistencies, in combination with data from addiction studies showing deficits in goal-directed behavior and preference for habit-based strategies (Ersche et al., 2016; McKim et al., 2016a), suggests further testing with this task in populations with special disorders to disentangle changes in neurobiological function that may result in abnormalities in action selection.

In contrast to the tasks described above, our lab utilizes a stimulus-response (S-R) task with deterministic response options mapped onto abstract visual images. Initial studies in humans employed this approach by mapping select image types directly on one key or another, similar to the option for animals to learn instrumental presses on a right or left lever. With the use of only 2-4 keys, human participants are able to learn such S-R associations rapidly (Deiber et al., 1997; Toni et al., 2001), which limits their utility for the investigation of learning time courses in neuroimaging studies or for repeated study sessions for treatment seeking psychiatric populations. Boettiger & D'Esposito (2005) developed an S-R learning task, the Hidden Association Between Images Task (HABIT), suitable for fMRI investigation of learning effects over time that include multiple task permutations to be used for investigating effects of interventions on S-R learning and re-learning. In this task, participants learn through trial and error feedback the correct motor responses (button presses) to abstract visual stimulus sets. Participants are trained in an initial session, and return for a testing session in which additional sets are learned, and a response devaluation manipulation is employed. This instructed response devaluation alerts participants that some S-R contingencies are changed, and allows measurement of habitual responding after changed contingencies for both well-established S-R sets versus freshly learned S-R associations. This novel paradigm provides advantages to measuring behavior that unfolds over time, includes task specific conditions in which general deficits in response inhibition can be controlled for post-

devaluation, and allows for assessment in multiple contexts, such as pharmacological therapeutic interventions, without practice effects that may confound results. We have previously demonstrated the utility of this task in individuals with an SUD history (McKim et al., 2016a), and will further test the applicability of this behavioral paradigm to manipulations of stress and tACs to modify habitual behavior.

Habitual Behavior in Addiction

Data from animal research investigating the role of drugs of abuse in potentiating habit-based responding has demonstrated mixed evidence for both goal-directed and habitual drug seeking behavior, with the relative contributions of distinct neural circuits to be dependent upon training and study parameters (Root et al., 2009; Gremel and Costa, 2013). For example, operant responses for alcohol and cocaine have both been shown to be goal-directed (Olmstead et al., 2001; Samson et al., 2004) or habit-based (Dickinson et al., 2002; Miles et al., 2003). The formation of habit-based responses in operant alcohol self-administration paradigms is facilitated by exposure to alcohol during concurrent operant training with a sweetened (sucrose) alcohol solution (Mangieri et al., 2012), or chronic exposure to ethanol vapor prior to self-administration of ethanol itself (Lopez et al., 2014). Moreover, home-cage access to alcohol prior to self-administration training facilitates habitual sucrose seeking (Corbit et al., 2012). Interestingly, cocaine administration after acquisition of responding and prior to devaluation can also shift responding for a natural reinforcer to be habit-based (Schmitzer-Torbert et al., 2014). Further support for the development of inflexible behavior resulting from alcohol exposure stems from nonhuman primate research in which access to alcohol over several years supports habitual alcohol seeking behavior that is controlled by environmental context, rather than action outcome (Cuzon Carlson et al.,

2011). Furthermore, studies on cocaine (Zapata et al., 2010), amphetamine (Nelson and Killcross, 2006), and intravenous nicotine self-administration (Clemens et al., 2014) in rodents show that chronic exposure to each of these three drugs of abuse produces a shift in operant behavior from goal-directed to habitual. Despite differences in drug type used and paradigm for training or administration, drugs of abuse can have potent effects on the development of habitual responding, and may even accelerate their development in specific circumstances. These studies provide a foundation for further study in humans, and suggest the need to examine the continuum of alcohol and drug use behavior, including individuals that use these substances/alcohol regularly without meeting criteria for addiction (heavy binge drinkers) as well as individuals meeting criteria for addiction and persons in recovery.

Direct translation of instrumental behavioral tasks used with animals have been applied to human studies of drug abuse and addiction to measure goal-directed and habitual behavior. An initial study investigating choice selection for probabilistic outcomes of cigarettes or chocolate were performed in young adult smokers (Hogarth and Chase, 2011). Participants consumed either chocolate or cigarettes prior to testing, i.e. outcome devaluation was employed, and behavior was found to be goal-directed toward the devalued outcome. This outcome was contrary to their expectation that cigarette-seeking wound be habitual, but may reflect the fact that severity and regularity of cigarette use was low in their sample, further indicating that severity of drug use can influence action selection. A later study by this group used chocolate and water as action outcomes, again with outcome-specific satiety to devalue each outcome, and demonstrated that non-daily smokers reduced responding to devalued outcomes (Hogarth et al., 2012a); however, individual differences depended in part on motor impulsiveness, as indexed by the Barratt Impulsivity Scale (BIS), such that greater motor impulsiveness was associated with less sensitivity to outcome

devaluation, and therefore more habit-based responding. A further study by this group that examined acute alcohol effects on behavioral action selection parallels that of animal studies (Corbit et al., 2012; Lopez et al., 2014) by providing exposure and self-administration of alcohol prior to behavioral testing. Using the same behavioral paradigm described previously with chocolate and water rewards, but only chocolate outcome devaluation, non-contingent alcohol administration facilitated a behavioral switch to habitual responding for the devalued reward after devaluation (Hogarth et al., 2012b).

Recent studies have begun to test goal-directed and habitual behavior in individuals meeting criteria for alcohol or other SUDs. For example, behavioral studies in alcohol-dependent subjects during abstinence demonstrated impairment in model-free behaviors using the two-step decision task (Sebold et al., 2014), whereas another study did not find differences relative to healthy controls in model-free behavior, but showed that abstinence was related with improvement in model-based control (Voon et al., 2015). This two-step decision task has also been used to test for habitual responding in people with methamphetamine addiction, where deficits were found in model-free (i.e. goal-directed) action selection (Banca et al., 2016). The task employing incongruent S-R associations and the slips of action paradigm were also recently tested in individuals with cocaine dependence, although most participants in this group met criteria for poly-substance dependence (Ersche et al., 2016). Results demonstrated deficits in learning in general, and that increased training resulted in outcome insensitivity in the SUD group. During the slips of action test, people with SUDs were more likely to respond to devalued stimuli relative to the control group. While that study found general deficits in learning, results from recent work in our lab using the HABIT over-training paradigm demonstrated that currently abstinent individuals who meet clinical criteria for dependence on one or more substances (including alcohol), perform

similarly to healthy controls on execution and learning of S-R associations (McKim et al., 2016a); the SUD group is specifically impaired in replacing responses to familiar stimuli after response devaluation. Relative to healthy controls, individuals in the SUD group showed increased perseverative errors for well-practiced S-R sets in which the response contingency changed, suggesting an inability to change automatized S-R associations. Regardless of task variations and substance of abuse, the data discussed above from human studies is beginning to mirror results from animal studies, potentially implicating drugs of abuse in potentiating habitual behavior above and beyond goal-directed actions specific to the context of drug use. An outstanding question is whether these changes in behavior are consequent to addiction or represent a pre-existing trait.

The underlying neural bases of the behavioral differences in goal-directed and habit-based responding are largely unknown, as are the neural bases of the deficits in attempts to change habitual responding that we observed among people with SUDs. However, both clinical and preclinical lines of research lend support to the idea that frontostriatal neural circuitry plays a key role. In humans, abnormal functioning of the OFC and striatum have been consistently found in people with SUDs (Ersche et al., 2005; Olausson et al., 2007; Park et al., 2010; Goldstein and Volkow, 2011; Konova et al., 2012). Decreased functional coupling between the PFC and striatum, and increased connectivity between the dorsal and ventral striatum, during resting state neuroimaging analysis has been shown in abstinent heroin addicts, which reveals that deficits in executive control over subcortical valuation systems may remain overactive in the drug free state (Xie et al., 2014). Additionally, lifetime experience with drug use is associated with an increased use of a S-R learning strategy, which is correlated with greater dorsal striatal volume in humans (Bohbot et al., 2013). Preclinically, in animal models of relapse, the DLPFC homolog, mPFC, appears to play a critical role in cue-induced drug self-administration (Jackson and Moghaddam,

2001; Feltenstein and See, 2008), as well as reinstatement of drug use after extinction (Kalivas and McFarland, 2003). Nonhuman primate work has also demonstrated that prolonged cocaine intake profoundly impairs S-R re-learning (Jentsch et al., 2002). Animal research into cocaine seeking using punishment (shock) concurrent with reward delivery has also demonstrated that the prelimbic cortex and the anterior DLS are important brain regions mediating habitual drug use behavior (Jonkman et al., 2012; Chen et al., 2013). Furthermore, the necessity of the prelimbic mPFC was demonstrated by optogenetic inhibition and excitation, such that excitation decreased cocaine seeking under shock conditions whereas inhibition of prelimbic mPFC increased punished cocaine-seeking behavior. In contrast, one human neuroimaging study has directly tested goal-directed and habitual behavior modeled from animal studies, with the S-R task employing incongruent trial types and instructed outcome devaluation, in alcohol dependent individuals. Results showed preferential use of habit-based responding (Sjoerds et al., 2013). This behavior was related to decreased vmPFC and increased posterior putamen activation during instrumental learning in the alcohol group compared to controls. Alcohol dependent individuals also showed greater activation of the posterior putamen relative to controls during response selection for incongruent trial types, in which it is most advantageous to use a habit-based response strategy. Together, the animal and human literature provide evidence for dysfunction within frontostriatal circuits in addiction that may perpetuate drug use behavior at the expense of goal-directed control; preferential strengthening of this circuit may decrease treatment efficacy and increase risk for relapse. Furthermore, triggers such as stress, which are known to target and modulate levels of PFC catecholamines (Arnsten, 2015), may also activate bottom-up circuits that encompass striatal brain regions, further biasing actions toward automatic, repetitive behaviors.

The Role of Stress in Facilitating Habitual Behavior

Alcohol and drug use may be motivated by a desire to increase the positive and rewarding effects of the drug, such as social interaction or mood elevation, or, alternatively, for negative reinforcement, such as decreasing negative affect or alleviating the stress/anxiety that may result from withdrawal. These outcome-based motivations are thought to be most prominent in driving drug-use or -seeking behavior to initiate the addiction cycle, when behavior may still be under control of the outcomes (goal-directed); however, if an individual has transitioned to regular drug use and then is able to remain abstinent, triggers such as stress can promote relapse, presumably through reactivation of both goal-directed and habitual neural circuitry that may ultimately compete for control of action selection. Therefore, stress may hypothetically promote relapse through a return to behavioral responses in an automatized manner (habitual) once drug use has been re-initiated. Stress has been shown to be a predictor of relapse behavior, but the underlying neurobiological mechanisms are not well understood (Sinha, 2012; Bossert et al., 2013; Sinha, 2013; Mantsch et al., 2016).

Human studies examining the role of stress in shifting behavior away from goal-directed control to preferential use of habit-based strategy in healthy controls have employed psychosocial and pharmacological methods to induce stress. Human behavioral studies of stress using the well-validated Trier Social Stress Test (TSST) demonstrated that this acute stress manipulation prior to learning in a virtual maze task resulted in the use of a S-R learning strategy (Schwabe et al., 2007). Further work by this group developed a socially evaluated cold pressor test (SECPT) that combines social-evaluation with the cold pressor task (CPT), hand immersion in ice water, to increase hypothalamic pituitary adrenal (HPA) axis response in addition to the strong sympathetic activation that occurs in response to the CPT. Participants were asked to hold their hand in ice

water for up to 3 minutes, and told they were being video-taped for facial expressions, while being monitored by an unfamiliar and unfriendly experimenter. In the first study to test the SECPT and instrumental learning for food rewards based on the task used by Valentin et al. (2007), participants underwent stress or a control condition and then trained on the learning task. Outcome devaluation then occurred through consumption to satiety, and testing occurred in extinction. Results showed that exposure to stress rendered behavior habitual through an increase in the number of responses to the devalued outcome (Schwabe and Wolf, 2010). As the results of that study could not rule out an effect of stress on learning versus extinction performance, a follow-up study alternatively administered the SECPT or control condition after learning and devaluation, but immediately prior to extinction testing. This study confirmed the habitual nature of responding (Schwabe and Wolf, 2011). Furthermore, to block the stress effect of the SECPT on habitual responding, propranolol, a beta-adrenergic antagonist, was administered prior to stress and devaluation (Schwabe et al., 2011b). Propanolol was found to prevent habitual responding to the devalued reward when tested during extinction, and suggests that targeting noradrenergic signaling may be an effective way to diminish the stress-induced shift away from goal-directed responding.

Further pharmacological investigations, in combination with fMRI, to investigate the neurobiological bases of a stress-induced shift toward habitual behavior suggest that the putative neuroendocrine mechanism of action is the combination of elevation in cortisol and noradrenergic activity. For example, using the food devaluation task discussed above (Valentin et al., 2007), the administration of hydrocortisone, a synthetic glucocorticoid, and yohimbine, an alpha-2 adrenergic receptor antagonist to stimulate noradrenergic activity, in combination prior to task training resulted in habitual responding to the devalued reward; this behavioral result was accompanied by reduced activation in the OFC, which was interpreted as reflecting deficits in registering changes

in valuation (Schwabe et al., 2012). That study also found that striatal brain areas implicated in habitual behavior, the caudate and putamen, demonstrated increased activation during training, whereas activity in the putamen during extinction was unaffected by the combination of hydrocortisone and yohimbine administration and did not correlate with changes in stress hormones. These data further support the impact of stress on PFC function that reflects preferential impairment of neural circuitry supporting goal-directed actions by the excess stress hormones, and manifests behaviorally as habitual responding.

Examination into real-world psychosocial stressors has also demonstrated changes in the neural structures associated with goal-directed and habitual association learning. Soares et al. (2012) tested action selection for food outcomes and fMRI correlates in medical students who had just completed their long preparation for medical residence selection examination versus medical students engaged in their usual academic activities. Subjects were tested again 6-7 weeks later to measure persistent changes in action selection and associated neural correlates over time. For the assessment immediately following chronic exam preparation stress, stressed participants continued responding for devalued rewards whereas for non-stressed controls, behavior was sensitive to outcome devaluation. Moreover, fMRI results demonstrated greater activity in the left putamen of stressed subjects relative to controls, and greater activation of the right caudate in controls compared to stressed subjects. Reassessment of the stressed subjects 6-7 weeks post-examination showed that their action-selection behavior was now sensitive to outcome devaluation (i.e. goal-directed) once the stress had subsided. At the neural level, results were inline with previous findings in healthy controls, such that subjects that were assessed 6-7 weeks after recovery from stress had greater right caudate activity during devaluation testing. Changes in brain structure were also examined following chronic stress and after recovery from stress, demonstrating that in the

stressed participants (relative to controls) the volumes of the caudate and the left mOFC were lower whereas that of the putamen was increased. When measured again after recovery from stress, the volumes of the caudate and mOFC increased, while the volume of the putamen showed a trend toward a decrease, suggesting that the neural changes associated with stress are transient and reversible with time if stress abates. These studies are in agreement with evidence from animal models of chronic stress, which have demonstrated atrophy in the mPFC and associative striatal areas along with hypertrophy in the DLS, which accompanied habit-based responding for the devalued action-outcome contingency (Dias-Ferreira et al., 2009).

In addition to the biological components of the stress response that may contribute to changes in behavior, individual differences in perceived life stress levels and in executive function, such as working memory capacity, may increase or buffer the effects of acute stress on response strategy, respectively. The two-step decision task to examine model-free and model-based action selection was used with the CPT to measure changes in choice behavior, showing that stressed subjects were more likely to use model-free action selection relative to performing model-based decisions (Otto et al., 2013). The researchers further demonstrated that working memory capacity was protective against the effects of acute stress on action selection strategy, with sustained model-based behavior after stress being most evident in individuals with greater working memory capacity. In another study with this task, male participants underwent the TSST, which did not uniformly impact model-based or model-free action selection; however, acute stress was more likely to impair model-based action-selection in individuals self-reporting high levels of chronic life stress (Radenbach et al., 2015a). Importantly, these studies demonstrate that variables assessing individual differences, and which may impact prefrontal function, can result in differences in behavior that support a shift from goal-directed to habit-based responding.

Despite the evidence discussed above that stress potentiates habitual responding, this has not been directly measured in addiction with behavioral paradigms assessing goal-directed and habitual responding. Our previous behavioral study did not assess measures of stress, but did find group differences between anxiety levels from questionnaire data, with individuals in the SUD group showing higher trait levels of anxiety (McKim et al., 2016a). We were not able to rule out stress effects per se on our behavioral findings, and this illustrates the need to include such assessments in future studies that test habitual responding in addiction. Furthermore, the stress effects observed from other studies examining goal-directed and habitual behavior have left several unanswered questions that are relevant to understanding these behaviors in general, as well as their application to studies of addiction. First, the importance of the timing of the SECPT stress manipulation on instrumental behavior acquisition versus task performance in general has not been resolved (Schwabe and Wolf, 2009, 2010); previous results from Schwabe et al. (2009) demonstrated that some participants were not able to learn the task after stress, and increases in cortisol persisted above baseline throughout testing, which limits specificity of behavioral task effects. Additionally, the effects of stress on behavior were more pronounced when healthy controls were stress prior to task learning as opposed to immediately prior to devaluation (after training) (Schwabe and Wolf, 2010). This suggests that timing is a key factor in predicting later behavioral deficits, and should be examined further in studies of addiction. The authors further speculate that the habitual nature of responding could result from ineffective cognitive control or response inhibition (Schwabe and Wolf, 2010). While deficits in response inhibition occur in addiction (Morein-Zamir and Robbins, 2015; Moeller et al., 2016), our previous study was able to rule out this alternate possibility with built in task controls to demonstrate the specificity of behavioral effects to perseverative errors (McKim et al., 2016a). We will expand our initial

behavioral study to manipulate stress timing in healthy controls to investigate the effects of stress on the execution of practice versus new learning of S-R sets and to examine whether stress results in deficits of re-learning or changing habit-based behaviors after response devaluation.

Non-Invasive Brain Stimulation as a Method to Probe Neural Circuit Control over Habits

The research discussed above provides evidence that frontostriatal circuitry is necessary for coordinating goal-directed and habitual behavior in both animal models and humans. Evidence of frontostriatal circuit dysfunction in addiction in general, in combination with direct evidence from goal-directed and habitual paradigms in SUD history individuals, indicate that interventions that are able to target this neural circuitry may be successful for treatment and the prevention of relapse. At the behavioral level, research findings demonstrate both deficits in goal-directed and habitual responding; these results may stem from different paradigms to test the underlying mechanisms and suggest that the changes in behavior observed could result from a loss of PFC control or a shift toward preferential striatal control over behavior. To date, one neuroimaging study has demonstrated that the former, deficits in PFC control over goal-directed behavior, resulted in habit-based responding. Whether this behavioral result could also emerge from strengthened striatal control relative to intact PFC function in persons with SUDs has not currently been demonstrated at the neural level in humans. To begin to assess and manipulate how the PFC may coordinate action-selection behavior, non-invasive brain stimulation methods such as TMS and tDCs have been used to measure changes in goal-directed versus habit-based strategies in healthy individuals. For example, theta burst TMS over the DLPFC during the two-step decision task shifted action-selection from a model-based to a model-free strategy (Smittenaar et al., 2013). Moreover, individuals with higher working memory capacity were less susceptible to the effects

of stimulation on action selection. A follow-up study by this group instead used tDCs to manipulate action selection in the two-step decision task, and showed that anodal stimulation of the right DLPFC did not change the use of either model-based or model-free action selection strategies (Smittenaar et al., 2014). These mixed findings demonstrate the need for further investigation into the effects of non-invasive brain stimulation techniques on action-selection.

The current state of knowledge on the effects of brain stimulation on goal-directed and habitual behavior is unclear, reinforcing the need for further study, particularly in special populations in which deficits in goal-directedness could be ameliorated by such stimulation. For example, a recent review on brain stimulation methods in addiction suggests that TMS of the DLPFC may be effective at reducing craving for substances of abuse, particularly nicotine (Salling and Martinez, 2016). Evidence for the effect of brain stimulation on misuse of other substances, including alcohol, is mixed, due to the smaller scope of studies and differences in targeted brain regions. To date, a few studies have employed tDCs in nicotine, alcohol, cocaine, and cannabis dependence, but the results are again inconclusive with increased variability dependent upon the electrode placement (brain area(s) targeted), substance of abuse, and methods of reporting effects of outcome variable of interest, such as craving, risking taking or motivation/affect in general. One promising stimulation technique is tACs, which utilizes sine wave current stimulation to modulate ongoing brain oscillations within a specific frequency range (Herrmann et al., 2013). tACs has recently been employed as a non-invasive technique to draw causal links between brain regions and cognitive functions (Herrmann et al., 2013). By modulating a target frequency in a particular brain area, tACs can synchronize activity or connectivity between disparate brain regions, and dependent upon phase stimulation parameters, may allow for decoupling of communication

between brain areas (Thut et al., 2012). These advantages reinforce the use of tACs to remedy aberrant neural circuit function is neuropsychiatric disorders such as addiction.

Although the use of tACs in modulating cognitive performance is relatively recent, preliminary evidence suggests that it may be an effective therapeutic method to target aberrant neural oscillation frequencies and communication between brain regions in neuropsychiatric disorders. To date, tACs has been used to perturb several aspects of cognition, including attention, perception, working memory, declarative memory, and cognitive control. Bilateral DLPFC tACs within theta frequency range (4.5 Hertz; Hz) increases working memory task performance in healthy adults (Meiron and Lavidor, 2014); when left DLPFC gamma (40 Hz) tACs was directly compared to tDCs stimulation on a working memory (*n*-back) task, only tACs stimulation facilitated performance in the highest load condition (Hoy et al., 2015). Bilateral DLPFC theta frequency (0.75 Hz) tACs did not alter declarative memory in a procedural task in older adults relative to previous findings in healthy younger adults, suggesting that stimulation parameters, participant population, and the specific task used are important study design considerations when testing tACs effects on behavior (Marshall et al., 2011; Eggert et al., 2013). DLPFC tACs can also enhance higher executive functions, such as fluid intelligence (Pahor and Jausovec, 2014), while tDCs stimulation of DLPFC can impair behavior on intelligence tests, in particular on perceptual reasoning, dependent on whether it includes unilateral or bilateral stimulation (Sellers et al., 2015). In contrast, alpha frequency (10Hz) tACs of the DLPFC enhances creative and abstract thinking (Lustenberger et al., 2015). Studies in the domain of cognitive control have tested the role of theta tACs on DLPFC function necessary for behavior. In a study on risky-decision making using the balloon analog risk task (BART), left DLPFC tACs, relative to sham or right DLPFC tACs, increased risky decisions (Sela et al., 2012). A recent study on reinforcement learning,

incorporating a reward-punishment reversal of learned response associations, found that bilateral DLPFC stimulation in the theta frequency resulted in better reversal learning, although individuals were more likely to use a less advantageous response strategy for reward (Wischnewski et al., 2016). There is even less published data on the effects of tACs in neuropsychiatric populations; the two studies of note have targeted declarative memory in children with ADHD, demonstrating improvement in performance after tACs was delivered to bilateral DLPFC during sleep (Prehn-Kristensen et al., 2014), whereas gamma tACs of left DLPFC did not enhance working memory performance in patients with schizophrenia in comparison to the improvement in performance found with left DLPFC tDCs in the same study (Hoy et al., 2016). The therapeutic benefit of non-invasive tACs is highly promising and has practical applicability in the real world; available parameters for current intensity and frequency can be tailored on an individual basis in combination with portability of the device outside of a clinic or laboratory setting. The mixed effects in stimulation studies (TMS and tDCs) in addiction demonstrate a need for interventions with more direct manipulation of neural circuit communication, in combination with behavioral paradigms to adequately test habitual and goal-directed action selection.

Goals of the Current Dissertation

Goal-directed and habit-based behaviors each rely on distinct frontostriatal circuits (de Wit et al., 2007; Kalivas, 2008; Kehagia et al., 2010; Noonan et al., 2011; Hadj-Bouziane et al., 2013). For example, lesion studies in both animals and human patients show the importance of frontostriatal circuits in the transition from goal-directed to S-R behaviors (Petrides, 1982, 1985, 1997; Murray et al., 2000; Naneix et al., 2009; Stalnaker et al., 2010). These are the same neural circuits that have been demonstrated to be abnormal in addiction (Goldstein and Volkow, 2011).

However, only one study to date has examined differences in these neural circuits with a S-R association paradigm in alcohol dependence (Sjoerds et al., 2013). Behavioral studies employing S-R paradigms in addiction suggest that the deficits in goal-directed behavior result from reduced PFC control over the striatum, but direct evidence at the neurobiological level is lacking. Furthermore, our study is the only one to date that has investigated the habit 'breaking' process in abstinent substance users; such knowledge is critical to understanding attempts to change habit-based responses to drug stimuli during recovery from addiction and thus promote relapse. The studies proposed within this research plan are designed to provide novel insight into the neurobiological processes that underlie S-R learning and re-learning impairments, with the potential to identify novel therapeutic interventions for SUDs.

We will use our HABIT behavioral paradigm in conjunction with a stress manipulation to potentiate habitual responding in healthy controls, while also separately testing non-invasive tACs of bilateral DLPFC to reduce habitual responding in persons with a history of an SUD. Our S-R task design uniquely allows elucidation of the effect of stress on attempts to change behavior during habitual responding. As demonstrated in Figure 1.1, our HABIT paradigm includes a Training (top panel A: 'Session 0') and a Test session (top panel A: 'Session 1') in which participants learn S-R associations during training and then practice these S-R sets ('FAMILIAR') intermixed with newly introduced S-R sets during Part 1 of testing ('NOVEL'); during this portion of testing, we can measure the preferential use of a goal-directed or habit-based response strategy over time. Participants are then informed that response contingencies for some of the learned S-R sets change. Part 2 of the testing session allows us to measure response selection strategy based on overall performance (accuracy) as well as the type of errors that participants commit. For example, we can measure habitual behavior in the form of perseverative errors, in which participants

continue to push the previously correct response for an S-R set with changed contingencies (Figure 1.1B). Our task also includes built in controls that include S-R sets that do not have changed response contingencies to control for context change and differences in positive versus negative feedback; we can also rule out deficits in responding that may be due to response inhibition (McKim et al., 2016a). We have previously demonstrated that individuals with a history of lifetime diagnosis of a SUD show deficits in perseverative errors after response devaluation relative to control subjects. Our HABIT paradigm will further enable the manipulation of stress timing during the test session to elucidate stress effects on learning versus re-learning S-R associations. We will also directly manipulate neural circuit function with bilateral DLPFC tACs to reduce perseverative responding in persons with an SUD history.

In the studies described in Chapters 2 and 3, we tested whether acute stress changes habit formation and adaptation in healthy control subjects, using the same behavioral S-R task in which we observed abnormalities in people with SUDs (McKim et al., 2016a). In Chapter 2, we tested the working hypothesis that stress impairs goal-directed learning, enhancing habitual responding in healthy young adult males. We used the SECPT to induce stress, which has been previously shown to enhance habitual actions and elevate physiological stress measures such as salivary cortisol and heart rate in healthy males (Schwabe et al., 2008; Schwabe and Wolf, 2010; Schwabe et al., 2011), to examine the effect of acute stress on learning versus overcoming habitual responses. We found that experiencing the SECPT at the beginning of the Test session (Fig. 2.1) impairs the ability to overcome habitual responding, as measured by the proportion of perseverative errors, a measure of the inability to change learned habit behaviors. Essentially, acutely stressed males performed similarly to individuals with an SUD history in our S-R task. Notably, this effect of the SECPT was not evident when males were stressed immediately prior to

response devaluation. As we describe in Chapter 2, the stress effects seem to depend on sympathetic activation, unopposed by counteracting parasympathetic activation. In Chapter 3 we further tested whether the effect of stress timing on habitual behavior varied across the menstrual cycle in females using the same methods as Chapter 2. In contrast to males, we did not find differences in stress timing on habitual responding in females; this effect was not dependent on menstrual cycle phase. Importantly, we demonstrate biological activation of HPA and sympathetic stress response to the SECPT, but these effects do not contribute to deficits in performance in females, supporting the necessity of examining gender differences and the role of sex hormones on cognitive tasks. Together, the studies described in Chapters 2 and 3 establish the stress sensitivity of the forms of learning assessed by our task, highlighting sex differences, and possible physiological mediators of the stress effects. These findings expand the utility of our task, and lay the groundwork for studies investigating interventions to reduce habitual responding, which could then be applied to clinical populations. Although this study population did not include those with SUDs, these results lend insight into how acute stress may serve as a potent relapse trigger in people with SUDs, as the mechanisms by which stress promotes a return to drug use remain unclear (Sinha, 2012; Sinha, 2013). Our findings may guide future studies that identify neurobiological mechanisms by which stress promotes relapse in addicts.

In addition to testing a manipulation designed to impair performance on our S-R learning task in healthy controls, the final study of this dissertation (Chapter 4) was designed to recover deficits in frontostriatal circuit function in individuals with an SUD history through the use of non-invasive tACs. Possible contributors to inflexible habits in individuals with SUDs are changes in the frontostriatal circuitry required for S-R execution and replacement. Therefore, our objective was to determine the sensitivity of goal-directed and habitual behavior to bilateral DLPFC tACs

stimulation in individuals with SUDs and in an age and gender-matched control group. Motivated by the work of our collaborator, Dr. Flavio Fröhlich, we selected a stimulation in frequency in the alpha range (10Hz), which facilitates creativity (Lustenberger et al., 2015). To achieve this aim, we tested the working hypothesis that any lifetime SUD diagnosis is associated with reduced top-down control during re-learning of established S-R associations, which could underlie impairments in the ability to change S-R associations (McKim et al., 2016a). Additionally, we predicted that in people with SUDs, active versus sham stimulation will reduce perseverative errors after response contingencies have changed for well-learned S-R associations, reflecting goal-directed action selection (McKim et al., 2016a). These hypotheses are motivated by our preliminary behavioral data showing that people with SUDs exhibit better performance in execution of S-R associations, but demonstrate deficits in flexibly changing S-R associations, particularly well-established associations (McKim et al., 2016a). To our surprise, we found increased perseverative errors during true stimulation relative to sham stimulation in the control group. In contrast, there were no clear effects of stimulation condition on perseverative errors in the SUD group. However, there was a trend for true stimulation to increase performance during responding for well-trained S-R sets. These results provide a foundation for future studies to test stimulation specificity within the PFC, and suggest the alternative explanation that alpha band tACs altered circuit level dynamics that resulted in impaired performance. Identification of specific brain oscillation frequencies that may be differentially altered by tACs between groups will provide essential information for future studies in which brain activity is manipulated and then measured via electroencephalography (EEG) or fMRI to test for causal roles in behavioral differences. Such findings will ultimately inform our testing of novel methods to facilitate habit eradication.

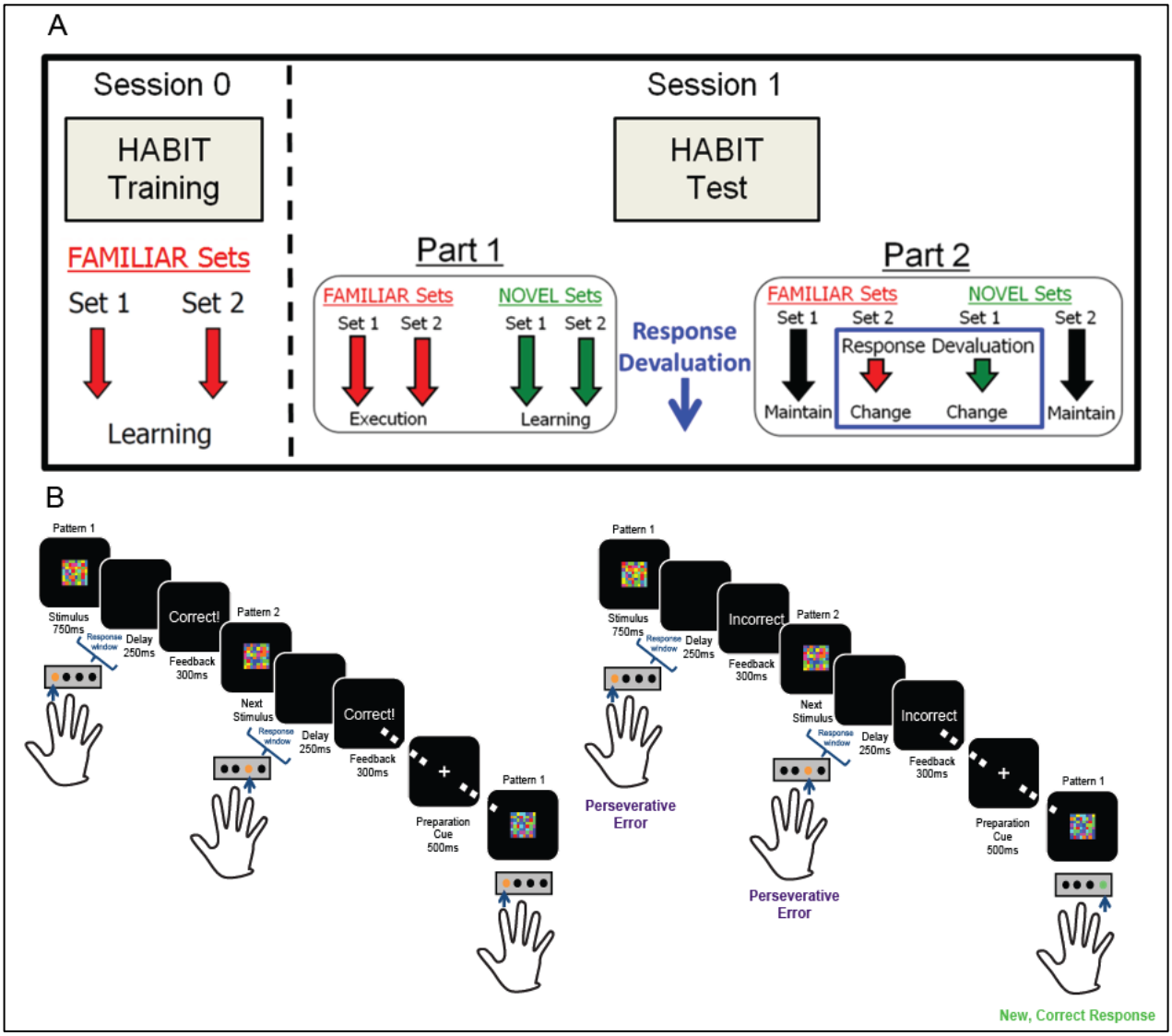

Figure 1.1. Schematic of HABIT paradigm. (A) Top panel depicts Training session ('Session 0') and subsequent Testing session on a separate day ('Session 1'). Session 1 is divided into Part 1 (pre-contingency change; six task runs) and Part 2 (post-contingency change; six task runs). (B) Bottom shows example images and responses for Part 1 (left; pre-contingency change) and Part 2 (right; post-contingency change).

CHAPTER 2

THE EFFECTS OF STRESS TIMING ON HABITUAL RESPONDING IN MALES

Introduction

The stress response is a naturally occurring biological reaction to psychological or physical threat and harm to the body. The initial and rapid response of the sympathetic nervous system (SNS) results in increased heart rate among other characteristic bodily effects encompassing the 'flight or fight' response. In contrast, the timing of HPA axis activation is slower relative to the SNS, but this system also releases neurotransmitters and hormones such as glucocorticoids, e.g. cortisol, that play an active role in the stress response. Importantly, these systems have dynamic effects over time that are regulated by negative feedback loops to return the body to normal homeostatic levels of activity and neurotransmitter/hormone balance once the stressor has subsided. However, prolonged or uncontrollable stress may increase susceptibility to various disorders, including the use of drugs or alcohol as a coping mechanism. For example, individuals with SUDs are hypothesized to have dysregulated HPA axis activity (Stephens and Wand, 2012), although it is unclear whether these changes within the system are a predisposition to, or a result from, drug or alcohol use behavior. Additionally, stress is a potent predictor of relapse behavior in SUDs, but the underlying biological mechanism of this effect are unclear (Sinha et al., 2011; Sinha, 2012). These data suggest that targeting the neurobiological underpinnings of stress may be potential treatment options and facilitate behavioral change strategies to prevent drug use behavior.

A promising area of research into the underlying biological mechanisms of stress-induced changes in addiction-relevant behaviors is that of goal-directed and habitual response selection

paradigms in humans. Shifts in action-selection toward a more habit-based behavioral strategy in SUDs (Sjoerds et al., 2013; Sebold et al., 2014; Banca et al., 2016; McKim et al., 2016a) is similar to behavioral findings in stress studies. For example, studies using a psychosocial stress manipulation, the SECPT, have demonstrated that stress prior to instrumental training, or after outcome devaluation (i.e. post-training) but prior to extinction testing, results in an increase in responding toward the devalued outcome relative to the non-devalued outcome, suggesting heightened habit-based actions in healthy males (Schwabe and Wolf, 2009, 2010). Moreover, administration of hydrocortisone and the α2-adrenoreceptor antagonist, yohimbine, to mimic biological stress effects increases habitual responding for a devalued instrumental outcome (Schwabe et al., 2012), although administration of either pharmacologic agent alone did not shift behavioral strategy. This research group further tested pharmacological blockade of SECPT stress effects prior to instrumental acquisition with propranolol, and demonstrated that blockade of noradrenergic activity resulted in a shift toward decreased responses to t devalued outcome, indicative of more goal-directed behavior (Schwabe et al., 2011b). These studies suggest that the putative neuroendocrine mechanism of action of the SECPT in healthy adult males is the combined elevation of cortisol and noradrenergic activity.

In contrast to evidence in humans on the underlying biological basis of stress on goal-directed and habitual behavior, only one rodent study to date has directly tested acute stress on instrumental response strategy. Rats exposed to acute restraint stress, or administered the combination of corticosterone and yohimbine, did not show insensitivity to outcome devaluation, and thus behavior remained goal-directed after sensory-specific satiety (Braun and Hauber, 2013); this does not replicate the human findings of hydrocortisone and yohimbine activation of a stress response and resulting habitual behavior. However, a more severe and multicomponent stressor

that included restraint stress in combination with tail shocks, exposure to an elevated platform, and loud sound and bright light stimuli, rendered behavior habitual after equivalent instrumental training to the single stress groups. Interestingly, the researchers tested plasma corticosterone levels in a separate cohort of animals exposed to one of the three stressors, but not undergoing instrumental training, which showed no difference between levels of corticosterone, and suggest a similar HPA axis response among the stress groups. Multiple facets of the stress response, in addition to the biological corticosterone response (cortisol in humans), may be important in determining whether physical versus psychosocial stressors can potentiate habitual responses for instrumental actions. These results highlight stressor severity as an important factor in regulating the shift between goal-directed and habitual responding.

Animal and human studies of chronic stress also demonstrate a bias toward habitual behavior. Chronic unpredictable stress in rats, including restraint stress, social defeat, and forced swim tests, biased behavior toward use of a habit-based strategy after devaluation and changes in outcome-contingency associations (Dias-Ferreira et al., 2009). Furthermore, rodents (mice and rats) administered a chronic regimen of corticosterone in drinking water, showed increased responding to devalued rewards after contingency degradation and outcome devaluation (Gourley et al., 2012). There are similar findings in human participants studied during ‘naturally’ occurring stress outside of the laboratory setting in medical students preparing to take medical residency exam (long preparation period) (Soares et al., 2012). Using the same instrumental learning task as the Schwabe group, participants were tested prior to taking the exam in a stressed state, and again 6-7 weeks after the exam in a non-stressed state. Results demonstrated reversible effects of chronic stress on behavior that went from more habit-based prior to stress, to goal-directed when tested again in a non-stressed state. These results demonstrate that the effects on behavior were transient,

illustrating that novel ways to cope with stress or change the use of these behaviors may have implications for preventing relapse in addiction, where stress is known to be a predictor in treatment outcome.

Taken together, the above data suggests that acute and chronic stress can result in shifts in behavior to habit-based responding. Whether this behavior coincides with a shift towards the predominate use of the brain regions governing habitual behavior, or instead merely reflects deficits in goal-directed circuit control over behavioral output that is habitual, is an outstanding question. Furthermore, existing study paradigms in humans have limited capacity to measure the development of habits over the time frame of multiple training days and test that are employed in rodent studies; this further confounds findings at the behavioral level, suggesting that the behaviors trained in the lab may not have transitioned to the ingrained behaviors that characterize disorders such as Obsessive Compulsive Disorder (OCD) and addiction. Results from human studies have also demonstrated that acute stress effects on habit behavior in the lab can occur regardless of the timing of the stressor (Schwabe and Wolf, 2009, 2010); these studies are not able to directly assess the effect of habit on goal-directed acquisition versus performance of habitual responding. Although this may be more readily studied in animal models, no studies to our knowledge have addressed this gap within the literature to draw parallels between species.

Here, we directly test the effects of SECPT timing on goal-directed and habitual responding in healthy adult males. To do so, we employed the HABIT paradigm (McKim et al., 2016a) to further understand the biological mechanisms of stress-induced shifts in instrumental behavior. Importantly, our task includes conditions in which stimulus-response (S-R) sets are well-practiced and over-trained, as well as the introduction of new S-R sets that require initial, goal-directed action-selection to learn the correct responses; this allows us to independently test the impact of

psychosocial stress on habit-based action-selection and goal-directed S-R learning behavior. Additionally, our task employs a novel response devaluation manipulation to quantify changes in habit-based responding while also ruling out effects attributable to generalize deficits in response inhibition following stress, which to our knowledge, has not been tested to date.

We used a between subjects design that included a training and separate testing session. Participants were trained on S-R sets during the initial (training) session and were randomized to one of three groups prior to the second session: SECPT stress prior to HABIT Test Part 1, SECPT stress prior to HABIT Test Part 2, or the no stress control group. We measured perseverative errors, the tendency to respond to the previously correct but now incorrect buttons after response devaluation. This allows us to quantify the response strategy participants employed when attempting to overcome highly trained versus newly acquired S-R associations. We also collected physiological measures of stress, including heart rate and saliva samples for cortisol and salivary α-amylase, and subjective ratings of the SECPT stress induction.

Methods

Participants

Healthy adult males were recruited from the University of North Carolina at Chapel Hill (UNC) campus and surrounding community via advertisements. Participants (n=53) were aged 18-40 years old with no known history of neurological disorders, no current psychiatric diagnoses or psychoactive drug or medication use (excluding moderate alcohol and caffeine), and an estimated IQ within the normal range (≥80). Four additional participants were recruited, but failed to return for or complete the testing session. Participants were asked to refrain from excessive caffeine intake (no more than their self-reported regular amount), and to refrain from physical exercise for

6 hours prior to the Test session. Each subject provided written informed consent as approved by the UNC Office of Human Research Ethics.

General Procedure

Subjects participated in 2 sessions, with at least 1 night's sleep between the first and second sessions. Subjects were either paid for their participation, including performance bonuses in the Test session, or participated for class credit; students that participated for credit were entered into a gift card drawing at the end of the semester, with more entries for greater accuracy during the Test session. During Session 0, participants completed a battery of standard questionnaires (see "Behavioral Inventories"), followed by behavioral training on the computerized S-R learning task (see "Behavioral Task"); no stress manipulation took place during Session 0. Based on literature suggesting that performance on the operation span (OSPAN1 working memory task predicts sensitivity to the SECPT (Otto et al., 2013), participants also completed the automated OSPAN (Unsworth et al., 2005). S-R Learning and habitual responding was then tested during Session 1. We used a between subjects design and participants were randomly assigned to one of three groups for the Test session: 1. Stress before HABIT Test Part 1; 2. Stress before HABIT Test Part 2; 3. No stress control. All sessions took place between the hours of 1200 and 1700 to control for the diurnal rhythm of cortisol secretion. The experimental procedure is illustrated in Figure 2.1.

Behavioral Inventories

We administered a number of standard questionnaires to quantify factors that could impact our results. We quantified alcohol use behavior with the Alcohol Use and Disorders Identification

test (AUDIT; Saunders et al., 1993) and substance use behavior with the Drug Use Screening Inventory, Domain I (DUSI-I; Tarter, 1990). We calculated density of familial alcohol abuse using the Family Tree Questionnaire (FTQ; Mann et al., 1985). Neuropsychological questionnaires included the Barratt Impulsivity Scale (BIS-11; Barratt, 1994), Rotter's Locus of Control scale (LOC; Rotter, 1966), the State-Trait Anxiety Inventory (STAI; Spielberger, 1985), the Thought Action Fusion scale (TAF; Shafran et al., 1996), the Connors' Adult ADHD Rating Scale (CAARS; Connors, 1997), and the Perceived Stress Scale (Cohen et al., 1983). Education and occupation were quantified with the Barratt Simplified Measure of Social Status (BSMSS) (BSMSS; Barratt, 2006). We estimated IQ with the Shipley Institute of Living Scale (SILS; Zachary, 1991).

Behavioral Task

The Hidden Association Between Images Task (HABIT) is a S-R learning and re-learning task implemented in E-Prime 2.0 (PST Inc., Pittsburgh, PA) comprised of a HABIT Training session and a two part HABIT Test session, which occurs on a subsequent day (Fig. 1.1). Task details have been described previously (Boettiger and D'Esposito, 2005; McKim et al., 2016a). In brief, abstract visual stimuli are presented on a color LCD screen, and subjects use a four-button keypad for manual response selection using the fingers of their dominant hand. Participants are given instructions and a brief familiarization prior to completing the Training session. Stimuli are displayed briefly (700 ms) on the screen, and participants learn through trial and error to associate stimuli with specific manual responses. During the Training session, participants learn two sets of S-R rules to a criterion of ≥ 90% accuracy (FAMILIAR sets). Participants then return to the lab after ≥1 night's sleep to complete the Test session. In the Test

session, participants first demonstrate retention of the previously learned (FAMILIAR) associations, then the learning task begins (HABIT Test Part 1; Fig. 2.1). In the learning task, blocks of the two FAMILIAR sets are interspersed with blocks composed of two new stimulus sets (NOVEL sets), to measure new S-R learning, and blocks of a control condition, consisting of novel, unrelated stimuli (No Rule set); blocks consist of 15 randomly selected stimuli from the relevant set. Following 6 "runs" of 15 blocks each (3 per set-type), subjects are informed that the correct responses for two sets (one FAMILIAR and one NOVEL set) have changed (HABIT Test Part 2; Fig. 2.1). As the previously correct responses for the changed sets produces a negative rather than positive outcome, one can construe this change in response contingency as a response "devaluation," which is not to be confused with outcome devaluation procedures traditionally used in studies of habitual responding (Dickinson, 1985). This "response devaluation" manipulation allows us to quantify habitual responding when attempting to overcome both well-learned (FAMILIAR) and freshly learned (NOVEL) S-R associations, as the proportion of perseverative errors can be taken as an index of the degree to which responses are outcome independent (i.e. habit-based), as opposed to outcome-driven (i.e. goal-directed). By introducing S-R changes for both FAMILIAR and NOVEL sets, at a point where performance is approximately equivalent, we can rule out performance deficits due to impaired response inhibition. Moreover, including FAMILIAR and NOVEL sets in which correct responses do not change allows us to control for effects on performance of time and of context change.

Stress Protocol

Participants in the stress groups (Stress before HABIT Test Part 1, n=19; Stress before HABIT Test Part 2, n=18; detailed below) were exposed to the socially evaluated cold pressor test

(SECPT), described in detail elsewhere (Schwabe et al., 2008). In brief, participants were ask to immerse their non-dominant hand up to and including the wrist into ice water (33°F) for up to 3 min. Participants were asked to face in the direction of and look toward two video cameras, and were told that the camera(s) would record their facial expressions during the immersion procedure. An unsociable and unfamiliar experimenter monitored participants during the SECPT. During the SECPT monitoring, the unsociable experimenter wore a white lab coat, and held a timer and a clipboard. Most of the unsociable experimenters employed in this study were female, but males were used in five cases. The gender distribution of experimenters did not differ between stress groups $\chi^2_{(1)}=0.29$, $p=0.61$), and we found no effect of experimenter gender on our index of habitual responding, FAMILIAR perseverative errors ($F_{(1,32)}=0.08$, $p=0.79$). Including experimenter gender as a covariate in our analyses did not qualitatively alter our effects of interest.

Participants were instructed that the immersion procedure was intended to measure their stress response, and that they were allowed to take their hand out of the water at any point, but that they should hold it in for as long as possible. The SECPT procedure was identical for both stress groups, but the timing of the SECPT during the Test session differed between groups to measure the impact of stress timing on behavior during the Test session (Fig. 2.1). Participants assigned to the control group immersed their non-dominant hand up to and including the wrist for 3 min in warm water (80°F), during which they were neither videotaped nor monitored by an unsociable experimenter. We measured water temperature to confirm a significant effect of group ($F_{(2,50)}=2330.04$, $p<0.001$); the water temperature for the stress before HABIT Test Part 1 group was significantly colder (32.75 ± 1.41°F) than for the control group (79.75 ± 3.30°F, $p<0.001$), with an equivalent difference between the stress before HABIT Test Part 2 group (33.01 ± 1.80°F) relative to the control group ($p<0.001$). Critically, water temperature did not differ significantly

between stress groups (p=1.00). To assess responses to the SECPT, we collected subjective ratings of the SECPT, along with biological measures of heart rate, salivary cortisol, and salivary α-amylase from all participants in each group.

Subjective Stress Ratings

Participants completed subjective ratings immediately after the SECPT or control condition; this consisted of a questionnaire rating the stressfulness, unpleasantness, and painfulness of the SECPT manipulation on a scale of 0 (not at all) to 10 (very much), based on Schwabe et al. (2008).

Heart Rate

To measure changes in autonomic nervous system function as a result of the SECPT, we measured heart rate at three time points during the Test session. To collect this data, participants cleaned their skin with an alcohol pad and dried the areas with gauze prior to the placement of disposable Ag-AgCl foam electrodes (Biopac Systems, Inc; Goleta, CA). Electrodes were placed according to the three lead system: below the right and left collarbone area, and below the left ribcage. Electrode signals were sent via a Bionomadix wireless transmitter to a Bionomadix RSPEC receiver/amplifier system (Biopac Systems, Inc), and collected in Acqknowledge 4.3 (Biopac Systems, Inc) with a sampling rate of 500 Hz.

Heart rate was collected three times during the Test session: 1) ~10 min after arrival to the lab, 2) during the SECPT, and 3) ~5 min after the termination of the SECPT. For the pre- and post-stress heart rate measures, we collected 5 min of heart rate at rest while the participant was sitting

in a quiet room at a desk. They were instructed to remain as still as possible to minimize noise artifact and were allowed to read magazines provided or remain seated at the desk with their eyes closed. The duration of heart rate collection during the SECPT varied, with a maximum duration of 3 min; heart rate was recorded for as long as the participant held their hand submerged in the water and the recording was stopped when they requested to remove their hand. Electrocardiogram data was visualized, cleaned for artifacts, and processed offline using Mindware 3.0 HRV software (MindWare Technologies, Ltd; Gahanna, OH) by a research assistant blind to group assignment.

Heart Rate Variability (HRV) Measures

The parasympathetic and sympathetic branches of the autonomic nervous system play a prominent role in the control of heart rate, providing a balance of cardiac activity through tonic parasympathetic inhibition that dominates over sympathetic input at rest (Ernst, 2014). The timescale of parasympathetic modulation is on the order of milliseconds, whereas sympathetic changes occur more slowly, on the timescale of seconds; beat-to-beat changes in the heart rate time series, or heart rate variability (HRV), thus represents vagal dominance driven by parasympathetic control over the inputs from the sympathetic nervous system (Saul, 1990). HRV is an important marker of healthy control of cardiac activity, as low HRV is associated with neuropsychological disorders and increased risk of mortality (Camm et al., 1996; Thayer and Lane, 2007; Thayer et al., 2009). To assess HRV changes in response to stress, and as a measure of recovery from the acute SECPT challenge, we used both time and frequency domain measures. Our time-domain measure is the most commonly used: the root mean square of successive differences (RMSSD) in the interbeat interval; we used the natural logarithm transformed RMSSD data for all analyses (lnRMSSD). In addition to this time-domain measure, we also used frequency domain analysis to

provide information about the amount of variance or power of the time series within the high frequency band (HF; 0.15-0.4 Hz), as the HF power band primarily reflects parasympathetically mediated input to the heart (Freedland and Steptoe, 2010; Thayer et al., 2012). For the frequency domain analyses, we report normalized units, by taking the raw millisecond squared values to calculate the following normalized units : HF/(LF +HF) (Burr, 2007).

Salivary Cortisol

We measured HPA axis activity, including stress reactivity, via salivary cortisol samples. We collected saliva samples in specialized collection tubes (Sardstedt Inc., Newton, NC) at six time points during the Test session, and samples were stored frozen until assayed. Saliva samples collection time points were as follows: 1) ~5 min after arriving to the lab; 2) ~5 min before the SECPT; 3) ~5 min after SECPT completion 4) 20 min after SECPT completion, when HPA stress response is expected to peak (Engert et al., 2011; Allen et al., 2014); 5) immediately prior to Test session Part 2 (response contingency change); 6) at the end of Test session Part 2 (end of study; see schematic in Fig. 2.1). Free cortisol concentrations were measured using an enzyme-linked immunosorbent assay (Salimetrics LLC, State College, PA); inter- and intra-assay coefficients of variance were both below 15%. All saliva samples within a participant were assayed in duplicate on the same plate. Following Miller et al. (2013), we defined "cortisol responders" as those individuals whose salivary cortisol concentration at 20 min post-SECPT was ≥15% higher than their baseline salivary cortisol concentration.

Salivary α-Amylase

Salivary α-amylase concentration provides a non-invasive measure of SNS activation, as salivary α-amylase secretion is controlled by direct sympathetic innervation of the salivary glands (Schumacher et al., 2013). Thus, to assay sympathetic response to the SECPT, we assayed α-amylase concentration in the saliva samples collected pre-stress (~5 min pre-SECPT) and post-stress, at both 5 and 20 min post-SECPT. Salivary α-amylase levels were determined using a kinetic enzyme assay protocol (Salimetrics). Inter- and intra-assay coefficients of variance were both below 5%.

Data Analysis

Our primary index of task performance was accuracy during the HABIT Test session. The HABIT is composed of 6 "runs" prior to response contingency change (Part 1), and an additional 6 runs after the contingency change (Part 2; Figure 2.1). We calculated accuracy in three time bins in Part 1 and 3 time bins in Part 2 by binning together 2 runs ("early", "mid", and "late") for each part. When sphericity assumptions were violated for repeated measures ANOVA analyses, we applied Greenhouse-Geisser corrections for degrees of freedom. We differentiated error types (perseverative button press, other incorrect button press) post-contingency change to uncover response-selection strategies utilized by participants. We also collected reaction time (RT) data in each trial. We tested for group differences in demographic and psychometric variables with one-way ANOVA for continuous measures and χ^2 tests for categorical measures. All post-hoc tests were Bonferroni corrected for multiple comparisons. All data analyses were performed in SPSS 22 (IBM) or SAS 9.4 (Cary, NC).

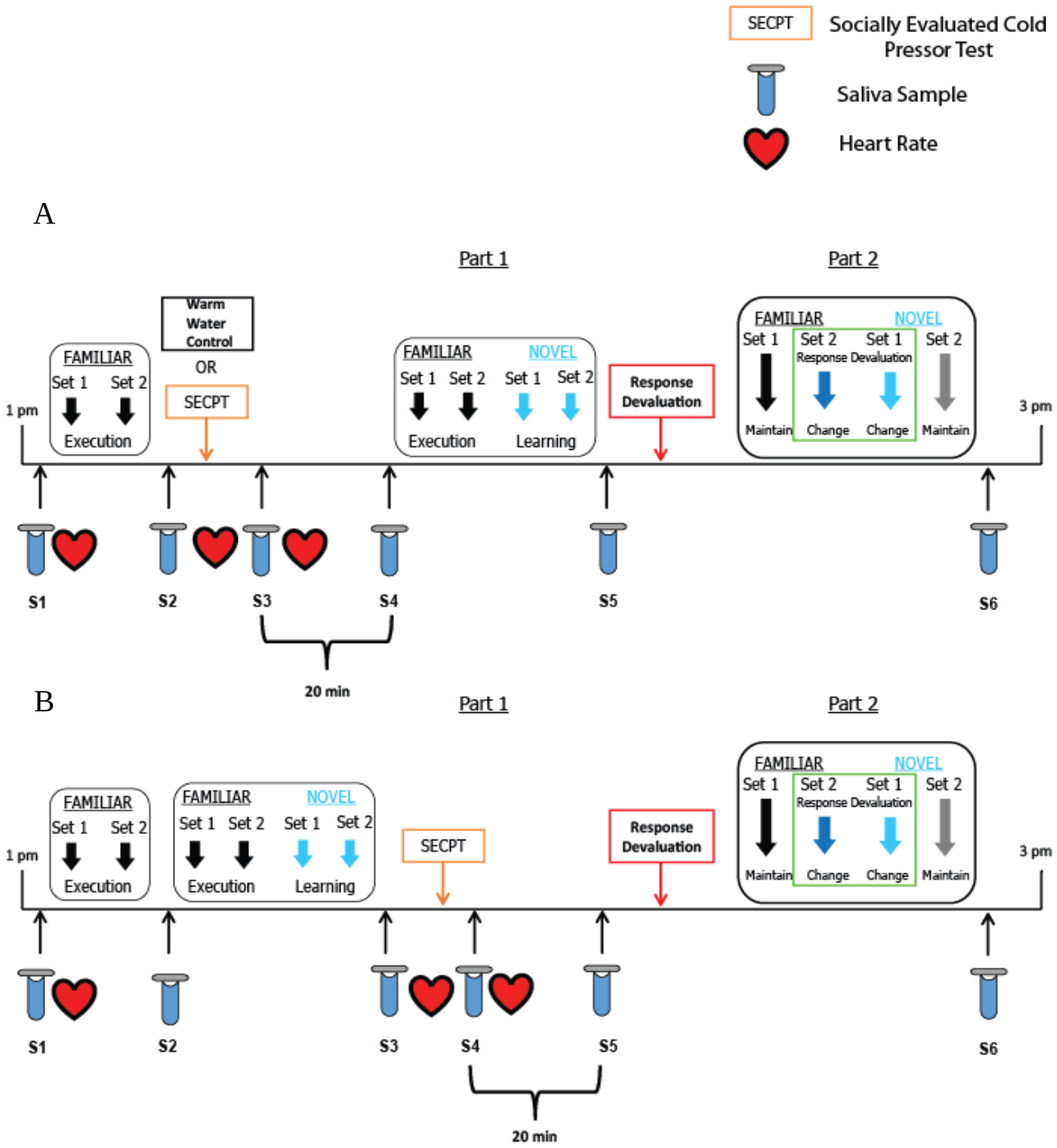

Figure 2.1. Schematic of Test session (session 1) protocol. (A) Top panel depicts the timing of the socially evaluated cold pressor test (SECPT) or warm water control condition, heart rate, and saliva sample collection for participants in the stress before HABIT Test Part 1 group. (B) Bottom panel depicts timing of the SECPT, heart rate, and saliva sample collection for participants in the stress before HABIT Test Part 2 group.

Results

Demographic and Psychometric Data

The three test groups did not differ in terms of education, SES, age, or ethnicity (Table 2.1). We also failed to detect any significant differences between groups in terms of psychometric variables (Table 2.1). Importantly, the groups were matched in terms of self-reported perceived stress, which can impact the response to an acute stressor (Radenbach et al., 2015b). Groups were also matched in terms of working memory measured via the automated OSPAN (all *p*'s>0.24; Table 2.1), another factor linked to stress sensitivity (Otto et al., 2013).

Behavioral Performance during HABIT Training

During the HABIT Training session, subjects were required to reach a performance criterion of 90% accuracy for each (FAM) set. The order of FAM sets was counterbalanced across participants and set order did not differ between groups, $\chi^2(2) = 2.88$, $p=0.24$. Training to criterion took ~25 min, with no significant differences between groups in the average number of training blocks (of 40 trials) needed to learn the first FAM set, (Stress before HABIT Test Part 1: 4 ± 2.5 blocks; Stress before HABIT Test Part 2: 5 ± 5 blocks; No stress control: 3 ± 1.5 blocks; $F_{(2,49)}=1.66$, $p=0.20$, $\eta^2=0.06$). Learning the associative rules for the second FAM set was always more rapid, and the required number of blocks again did not differ between groups (Stress before HABIT Test Part 1: 2.5 ± 1 blocks; Stress before HABIT Test Part 2: 2 ± 1 blocks; No stress control: 2 ± 1 blocks; $F_{(2,49)}=0.18$, $p=0.84$, $\eta^2=0.01$). Participants were then required to reach 70% accuracy in a third practice version of the task that switched between FAM sets 1 and 2. Groups did not differ in the number of trials to reach this criterion ($F_{(2,50)}=1.09$, $p=0.34$). Thus, training performance between groups was equivalent prior to returning for the HABIT Test session. Additionally, the number of days that elapsed between the HABIT Training and Test sessions did

not differ between groups (Stress before HABIT Test Part 1: 6 ± 3 days; Stress before HABIT Test Part 2: 8 ± 6 days; No stress control: 11 ± 16 days; $F_{(2,50)}$=1.19, p=0.31, η^2=0.05). Participants demonstrated retention of previously learned FAM sets by again reaching the performance criterion of 70% at the start of the Test session by repeating the practice version of the task that switched between FAM sets 1 and 2. Groups did not differ in the number of trials to reach this criterion ($F_{(2,50)}$=0.47, p=0.63).

Table 2.1. Sample Demographics and Psychometric Data

	Group 1 Stress before Part 1	Group 2 Stress before Part 2	Group 3 No stress control	$F_{(2,50)}$	*p*-value
Demographics	(*n*=19)	(*n*=18)	(*n*=16)		
Age (yrs)	20 ± 2	21 ± 5	21± 4	0.52	0.60
SILS (calculated) IQ	107 ± 5	107 ± 5	105 ± 6	0.57	0.57
Education (yrs)	14 ± 1	14 ± 1	14 ± 2	0.56	0.58
SES	19 ± 10	19 ± 9	19 ± 9	0.007	0.99
Ethnicity (% non-white)	16	22	38	0.02	0.24[#]
Substance Use related					
AUDIT Total	2 ± 3	5 ± 4	4 ± 5	1.76	0.18
Consumption	2 ± 3	4 ± 3	2 ± 2	1.38	0.26
Dependence	0	0.39 ± 0.61	0.50 ± 1	2.55	0.088
Harm	0.32 ± 0.67	1.11 ± 1.53	0.75 ± 1.95	1.40	0.26
DUSI-I (%)	0.04 ± 0.08	0.15 ± 0.16	0.14 ± 0.19	3.14	0.052
FTQ density (%)	0.09 ± 0.11	0.18 ± 0.21	0.12 ± 0.11	1.58	0.22
Psychometric					
Perceived Stress	14 ± 6	15 ± 5	15 ± 7	0.08	0.93
BIS Total	59 ± 10	62 ± 12	60 ± 10	0.41	0.67
Attention	17 ± 4	18 ± 4	16 ± 4	0.69	0.50
Motor	21 ± 3	22 ± 5	21 ± 4	0.53	0.59
Non-planning	22 ± 5	23 ± 7	23 ± 5	0.23	0.79
LOC	10 ± 3	9 ± 3	11 ± 4	1.60	0.23
STAI-State Anxiety	40 ± 10	41 ± 9	41 ± 13	0.10	0.91
STAI-Trait Anxiety	35 ± 9	36 ± 10	37 ± 10	0.19	0.83
TAF Total	18 ± 15	15 ± 6	19 ± 14	0.52	0.60
Moral	17 ± 13	12 ± 6	15 ± 12	0.92	0.41
Self	1.05 ± 2.39	2.0 ± 2.40	2.75 ± 2.96	1.91	0.16
Others	0.11 ± 0.46	1.06 ± 1.66	1.12 ± 2.16	2.45	0.10
Connors ADHD Scale					
DSM Inattention	7.74 ± 4.28	9.33 ± 4.63	9.36 ± 4.38	0.78	0.46
DSM Hyperactivity	7.00 ± 4.00	9.00 ± 4.80	7.43 ± 4.00	1.10	0.34
DSM ADHD	14.74 ± 7.23	18.33 ± 7.61	16.79 ± 6.77	1.15	0.33
Working Memory					
OSPAN Score	44.17 ± 17.61	51.28 ± 14.08	46.19 ± 16.54	0.93	0.40
OSPAN Total	58.17 ± 10.89	63.78 ± 9.67	61.50 ± 8.21	1.52	0.23
Accuracy Errors	3.72 ± 2.84	5.50 ± 4.66	4.44 ± 1.90	1.26	0.29
Math Errors	5.17 ± 3.00	6.11 ± 4.87	5.88 ± 2.45	0.33	0.72
Speed Errors	1.44 ± 1.34	0.67 ± 1.00	1.44 ± 2.00	1.66	0.20

Values are reported as mean ± standard deviation. Reported *p*-values reflect the results of ANOVA. IQ, Intelligence Quotient; SES, Socioeconomic Status; AUDIT, Alcohol Use Disorders Identification Test; DUSI-I, Drug Use Screening Inventory, Domain I; FTQ, Family Tree Questionnaire;, Barratt Impulsivity Scale; LOC, Locus of Control; SILS, Shipley Institute of Living Scale; STAI, State-Trait Anxiety Inventory; TAF, Thought Action Fusion Scale. ADHD, Attention Deficit Hyperactivity Disorder; DSM, Diagnostic and Statistical Manual. [#]*p*-value represents result of Fischer's exact test.

Subjective, Endocrine, and Autonomic Responses to Stress

We measured stress induction via the SECPT using subjective stress ratings, as well as measures of salivary cortisol, salivary α-amylase, heart rate, and heart rate variability.

Subjective Stress Ratings

We observed a significant effect of group on the amount of time the participants held their hand in the water bath ($F_{(2,50)}$=9.61, p<0.001), which was driven by significantly shorter times in both the stress before HABIT Test Part 1 group (111.45 ± 58.66 s, p<0.001), and the stress before HABIT Test Part 2 group (117.18 ± 61.61 s, p<0.001) relative to the control group (180 s). Critically, duration of ice water immersion did not differ between the two stress groups (p=1.00). Thus, the two stress groups experienced equivalent cold pressor effects.

Another indication of the equivalent effectiveness of the SECPT in inducing stress comes from the subjective ratings data. One-way ANOVAs detected significant effects of stress group on reported stressfulness ($F_{(2,52)}$=36.68, p<0.001), unpleasantness ($F_{(2,52)}$=38.33, p<0.001), and painfulness ($F_{(2,52)}$=18.61, p<0.001). Post-hoc tests demonstrated that both stress groups found the hand immersion more stressful, unpleasant, and painful than did the control group (Table 2.2). Moreover, the stress groups did not differ significantly in terms of their subjective ratings on any dimension (Table 2.2).

Table 2.2. No Significant Differences Reported between Subjective Stress Rating and SECPT Parameters Based on Stress Timing

	Group 1 Stress before Part 1	Group 2 Stress before Part 2	Group 3 No stress control
Subjective Rating	(*n*=19)	(*n*=18)	(*n=16*)
Unpleasant	7.11 ± 2.42	7.17 ± 2.01	1.75 ± 1.57[#]
Painful	4.68 ± 2.36	4.50 ± 2.26	0.81 ± 1.38[#]
Stressful	6.16 ± 2.34	5.83 ± 2.57	0.44 ± 1.26[#]
SECPT parameters			
Time in Water (secs)	111.46 ± 58.66	117.18 ± 61.61	180[#]
Water Temperature (°F)	32.75 ± 1.41	33.01 ± 1.80	79.75 ± 3.30[#]

Values are reported as mean ± standard deviation. Reported *p*-values reflect the results of one-way ANOVA between groups. [#]*Indicates significant difference relative to the stress before Part 1 group resulting from Bonferroni corrected post-hoc tests.*

Salivary Cortisol

We detected no significant differences between groups in baseline salivary cortisol prior to the SECPT/control immersion (~5 and ~25 min after HABIT Test session start; all *p*'s >0.30 Fig. 2.1). To quantify the change in salivary cortisol as a result of hand immersion, we first averaged the cortisol concentrations of the two baseline samples, then subtracted this baseline value from the cortisol concentration in post-immersion samples. Salivary cortisol levels changed significantly over time ($F_{(2.37,118.48)}$=3.50, p=0.03, η^2=0.04), such that cortisol values rose post-stress, and declined by the end of the session (Fig 2.2). These time effects differed by group (time×group interaction: $F_{(4.74,118.48)}$=12.68, p<0.001, η^2=0.32), reflecting significant increases in salivary cortisol 20 min after the SECPT for both stress groups. Specifically, cortisol levels increased significantly more in the SECPT before HABIT Test Part 1 group (3.00 ± 4.40 nmol/L) relative to the no stress control group (-1.44 ± 1.93 nmol/L, p=0.001) at the 20 min peak time point. Salivary cortisol levels also increased significantly more in the SECPT before HABIT Test Part 2 group (3.22 ± 6.17 nmol/L), relative to the no stress control group (-1.59 ± 2.24 nmol/L,

p=0.03) at this time point. We note that salivary cortisol levels at the end of the HABIT Test session did not differ between groups (p=0.49). Together, these data indicate that the SECPT effectively induced this biological indicator of stress in both stress groups.

In addition to measuring changes in salivary cortisol for all individuals within the sample, following the method of Miller et al. (2013), we also separately evaluated 'stress responders' based on salivary cortisol response (see Methods). The proportion of cortisol responders did not differ between stress groups (stressed before HABIT Test Part 1, n=12/19; stressed before HABIT Test Part 2, n=11/18; $\chi^2_{(1)}$=0.02, p=0.90). Moreover, baseline cortisol levels showed no significant main effects of stress group, cortisol responder status, nor their interaction (min p=0.28), suggesting no appreciable difference in either baseline cortisol or cortisol reactivity between stress groups.

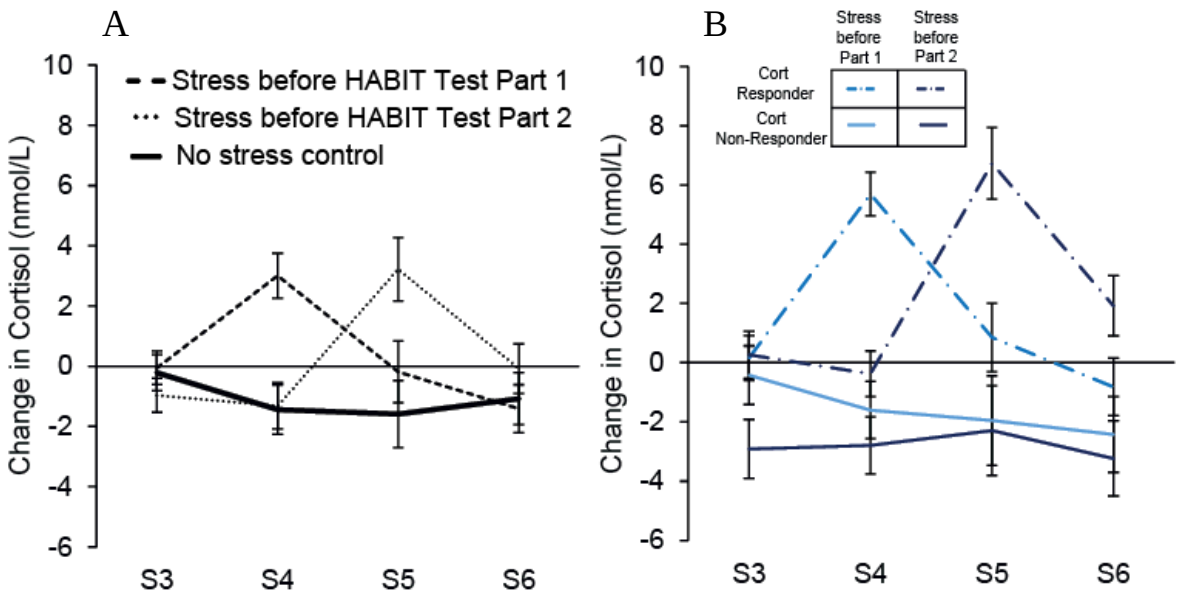

Figure 2.2. Salivary cortisol change over time relative to baseline cortisol average. Plot illustrates the time course of the change in salivary cortisol values (nmol/L) as a function of stress group and cortisol responder status. (A) Solid lines represent the control group, dashed lines represent the stress before HABIT Test Part 1, and dotted lines represent the stress before HABIT Test Part 2. Significant changes in cortisol (time×group interaction: $F_{(4.74,118.48)}$=12.68, p<0.001, η^2=0.32) measured at 20 min post stress for males stressed before HABIT Test Part 1 (S4; 3.00 ± 4.40 nmol/L, p=0.001) and males stressed before HABIT Test Part 2 (S5; 3.22 ± 6.17 nmol/L, p=0.03) show significant increases relative to the control group at S4 (-1.44 ± 1.93 nmol/L) and S5 (-1.59 ± 2.24 nmol/L). Cortisol levels at baseline (S3) and at the end of the study session (S6) did not differ between groups (p's>0.30). (B) Dashed lines represent cortisol responders and solid lines indicate cortisol non-responders. Light blue lines depict males stressed before HABIT Test Part 1 and dark blue lines depict males stressed before HABIT Test Part 2.

Heart Rate Measures

Heart Rate data

Heart rate data was collected at three time points during the HABIT Test session to measures changes over time. We collected heart rate ~5 min after arrival to the lab, during hand immersion, and ~5 min post-immersion (Figure 2.1). A repeated measures ANOVA detected a significant main effect of time ($F_{(1.72,77.41)}$=54.95, p<0.001, η^2=0.48; Figure 2.3), with planned comparisons demonstrating that heart rate increased during immersion ($F_{(1,45)}$=9.78, p<0.05) and then significantly declined by ~5 min post-immersion ($F_{(1,45)}$=105.21, p<0.001). As expected, we observed a significant time by group interaction ($F_{(3.44,77.41)}$=7.50, p<0.001, η^2=0.13; Fig. 2.3), which reflects significant group differences in heart rate during immersion ($F_{(2,50)}$=3.37, p=0.043) and 5 min post-immersion ($F_{(2,52)}$=4.14, p=0.02, but not at baseline ($F_{(2,49)}$=0.36, p=0.70). These group differences reflect that fact that the group stressed before HABIT Test Part 1 showed a significant increase in heart rate during immersion (89.84 ± 16.65 beats per minute (BPM)) relative to the group stressed before HABIT Test Part 2 (77.85 ± 13.00 BPM, p=0.047) but not the no stress control group (80.81 ± 12.85 BPM, p=0.24; Fig. 2.3). The group stressed before HABIT Test Part 2 did not show significant increases in heart rate relative to the control group (p=1.00). Moreover, at 5 min post-immersion, males stressed prior to HABIT Test Part 1 (71.14 ± 9.75 BPM) were not significantly different relative to the stress before HABIT Test Part 2 (65.00 ± 11.17 BPM, p>0.05) or the no stress control group (74.90 ± 9.45 BPM, p>0.05). However, males stressed before HABIT Test Part 2 had significantly lower heart rate relative to the control group (p<0.05) after stress. Qualitatively similar findings were present when only the cortisol responders were considered (data not shown). These heart rate data suggest that the group stressed before HABIT Test Part 1 experienced significant sympathetic activation, while the group stressed before HABIT Test Part

2 mounted a parasympathetic defense against the SECPT. We explored this possibility further using HRV and α-amylase analyses, below.

Heart rate variability (HRV) measure I: RMSSD

To probe parasympathetic activity, we first quantified variance in the interbeat interval (IBI) of successive heart beats as changes in the RMSSD from the same data used to calculate heart rate; data were natural log transformed due to violations of normality for raw RMSSD values (Shapiro-Wilk test: p's<0.016). A repeated measures ANOVA found significant main effects of both time ($F_{(2,90)}$=9.13, p<0.001 η^2=0.16) and group ($F_{(1,45)}$=3.79, p=0.03, η^2=0.17) on lnRMSSD (Fig. 2.3B). The time effect was driven by an increase in lnRMSSD from the immersion to post-immersion epochs ($F_{(1,45)}$=15.30, p<0.001). As is evident in Figure 2.3B, the group effect was due to higher lnRMSSD in the group stressed before HABIT Test Part 2 (4.94 ± 0.13), relative to the control group (3.54 ± 0.15, p=0.04); we observed a trend toward group×time interaction ($F_{(4,90)}$=2.19, p=0.076, η^2=0.07).

Heart rate variability (HRV) measure II: HF

In addition to evaluating heart rate variability in the time-domain, we also used frequency domain analysis to quantify power in the high frequency band (HF; 0.15-0.4 Hz), which primarily reflects parasympathetically mediated input to the heart (Freedland and Steptoe, 2010; Thayer et al., 2012). A repeated measure ANOVA found no main effect of time ($F_{(2,86)}$=1.36, p=0.265) nor a time by group interaction ($F_{(4,86)}$=1.05, p=0.39); however we did detect a substantial main effect of group ($F_{(2,43)}$=5.39, p<0.05, η^2=0.25). As is apparent in Figure 2.3C, this finding reflects substantially greater HF power in the group stressed before HABIT Test Part 2 (0.40 ± 0.03), relative to both the stressed before HABIT Test HABIT Test Part 1 group (0.30 ± 0.03, p=0.046), and the control group (0.26 ± 0.04, p=0.013). The group stressed before HABIT Test Part 1 and

the control group did not differ from each other in terms of HF power (p=1.00). Qualitatively similar findings were present when only the cortisol responders were considered (data not shown).

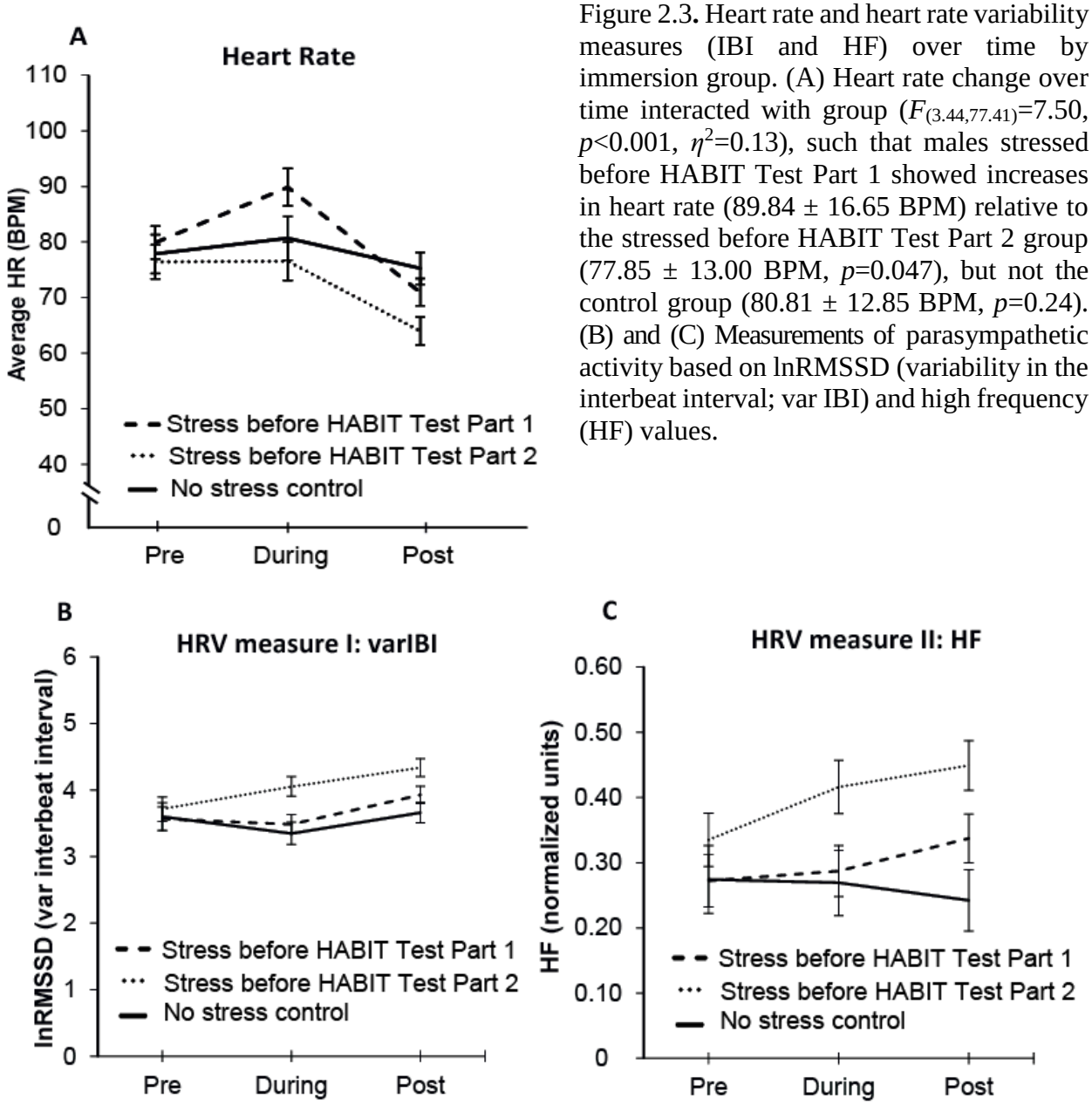

Figure 2.3**.** Heart rate and heart rate variability measures (IBI and HF) over time by immersion group. (A) Heart rate change over time interacted with group ($F_{(3.44,77.41)}$=7.50, p<0.001, η^2=0.13), such that males stressed before HABIT Test Part 1 showed increases in heart rate (89.84 ± 16.65 BPM) relative to the stressed before HABIT Test Part 2 group (77.85 ± 13.00 BPM, p=0.047), but not the control group (80.81 ± 12.85 BPM, p=0.24). (B) and (C) Measurements of parasympathetic activity based on lnRMSSD (variability in the interbeat interval; var IBI) and high frequency (HF) values. (B) Significant effects of time ($F_{(2,90)}$=9.13, p<0.001 η^2=0.16) and group ($F_{(1,45)}$=3.79, p=0.03, η^2=0.17) demonstrated that males stressed before HABIT Test Part 2 had higher var IBIs relative to the control group (p=0.04). (C) A significant main effect of group again demonstrated greater HF power in the males stressed before HABIT Test Part 2 (0.40 ± 0.03), relative to both the stressed before HABIT Test Part 1 group (0.30 ± 0.03, p=0.046), and the control group (0.26 ± 0.04, p=0.013).

Salivary α-Amylase

As a means of assessing sympathetic activation, we analyzed changes in salivary α-amylase at 5 min and 20 min post-immersion, anticipating a rise in salivary α-amylase at 5 min in the stressed groups, with weaker effects at 20 min. However, a repeated measure ANOVA detected no main effects of time ($F_{(1.65, 80.74)}$=0.42, p=0.66, η^2=0.01) or group ($F_{(2,49)}$=0.65, p=0.53, η^2=0.03; data not shown), and no interaction between time and stress group ($F_{(3.30, 80.74)}$=1.37, p=0.25, η^2=0.05). Qualitatively similar results were obtained when cortisol responder status was included in the analysis (all F's<1.67, p's>0.20).

Behavioral Results

Stress Effects on Learning New Sets and Execution of Familiar S-R Sets

To assess performance pre-contingency change, we conducted a mixed model repeated measures ANOVA with set-type (FAM/NOV) and time (early, mid, late) as within subject factors, and stressed prior to Part 1 (n=19) versus not (n=34), as the between subjects factor. We found expected main effects of set-type, with higher accuracy for FAM versus NOV sets ($F_{(1,51)}$=113.38, p<0.001, η^2=0.67), and time, with accuracy improving from early (0.66 ± 0.01) to mid (0.78 ± 0.01) to late (0.81 ± 0.01) runs ($F_{(1.72,87.86)}$=130.04, p<0.001, η^2=0.71; Fig. 2.4). We also found a significant set-type×time interaction ($F_{(2,102)}$=84.80, p<0.001, η^2=0.61), reflecting a greater improvement of NOV set performance over time (Fig. 2.4). To decompose the significant interaction between set-type and time, we ran separate repeated measures ANOVAs to evaluate time and group for each set-type. Performance improved over time for the FAM sets ($F_{(1.77,90.32)}$=20.54, p<0.001), and this improvement did not differ between stressed and unstressed participants ($F_{(1.77,90.32)}$=0.32, p=0.72). Interestingly, there was also a trend for an effect of group

on FAM set performance ($F_{(1,51)}$=3.40, p=0.071), likely reflecting ~3% more accurate performance in FAM sets prior to contingency change for the stressed group (Fig 2.4, far left panel). Consistent with our prior data, accuracy increased significantly with time for the NOV sets ($F_{(1.73,88.39)}$=167.12, p<0.001). We detected no main effect of group on NOV set learning ($F_{(1,51)}$=0.05, p=0.82), but did find a marginal interaction between time and group ($F_{(1.73,88.39)}$=3.09, p=0.057), which reflects a steeper initial improvement in accuracy in the stressed group relative to the unstressed group ($F_{(1,51)}$=5.02, p<0.001; Fig 2.4). Altogether, these data indicate that SECPT exposure slightly enhances performance of established S-R associations and acquisition of new S-R associations.

We further assessed changes in performance in terms of RTs, conducting an identical ANOVA to that described above, but taking RT as the dependent measure. We found a significant main effect of set-type ($F_{(1,51)}$=9.55, p=0.003), with slower RTs for NOV sets (520 ± 6ms) relative to FAM sets (511 ± 6ms). There was also a significant set-type×time interaction ($F_{(1.59,81.09)}$=7.84, p=0.002), suggesting that RTs for FAM sets decreased over time compared to RTs for NOV sets. Interestingly, we found a significant effect of group ($F_{(1,51)}$=4.71, p=0.035), showing that stressed participants had slower RTs (528.43 ± 10ms) relative to non-stressed participants (502.68 ± 7ms). Taken together, our results from acute stress in males demonstrate slower RTs and increased task accuracy.

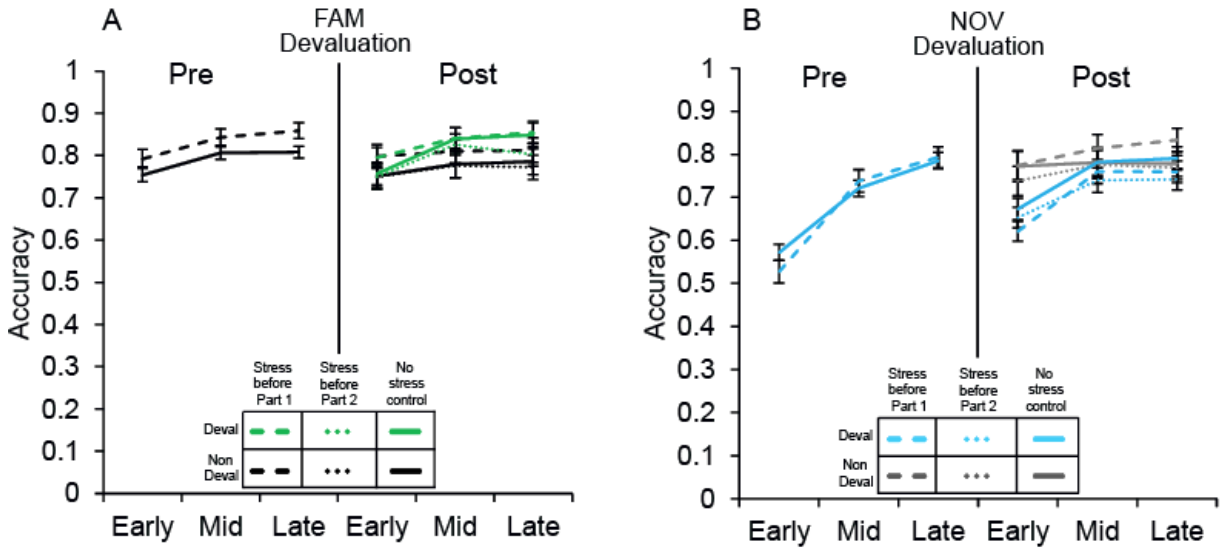

Figure 2.4. Accuracy performance for FAM and NOV sets before and after response devaluation by stress group. For accuracy prior to devaluation (left panels of A and B), we found a significant set-type×time interaction ($F_{(2,102)}$=84.80, p<0.001, η^2=0.61), reflecting greater improvement in NOV set accuracy over time regardless of stress. Performance improved over time for the FAM sets ($F_{(1.77,90.32)}$=20.54, p<0.001) for both groups, and there was a trend for better FAM set performance in the stress group (dashed line; $F_{(1,51)}$=3.40, p=0.071). For NOV sets, we found a marginal interaction between time and group ($F_{(1.73,88.39)}$=3.09, p=0.057), which reflects a steeper initial improvement in accuracy in the stressed group (dashed line) relative to the not stressed group (solid line; $F_{(1,51)}$=5.02, p<0.001). Accuracy performance post-devaluation (right panels of A and B) demonstrated significant interactions between contingency change and time ($F_{(2,100)}$=18.86, p<0.001) and set-type and time ($F_{(2,100)}$=3.44, p<0.05), indicating significant changes in performance between FAM versus NOV sets. A significant set-type×contingency change interaction ($F_{(1,50)}$=43.97, p<0.001) showed that performance on FAM sets was higher overall for the set with changed response contingencies (green lines; 0.81 ± 0.01) relative to the set that did not (black lines; 0.78 ± 0.02). In contrast, for NOV sets, overall accuracy was lower for the set with changed response contingencies ("Deval", blue lines; 0.73 ± 0.01) relative to the unchanged set ("Non Deval", grey lines; 0.78 ± 0.02). There were no significant effects of group ($F_{(1,50)}$=0.58, p=0.57) or group interactions (all F's<1.69, p's>0.16).

Behavioral Performance in Part 2 for Familiar and Novel S-R sets

To evaluate task performance post-contingency change, we first conducted a mixed model ANOVA with within subject factors of set-type (FAM or NOV set), contingency change (yes or no), and time (early, mid, late), and immersion group as the between subjects factor. We detected significant main effects of set-type ($F_{(1,50)}$=29.24, p<0.001, η^2=0.06) and time ($F_{(2,100)}$=45.74,

$p<0.001$, $\eta^2=0.10$), reflecting higher accuracy in FAM sets (0.80 ± 0.01) versus NOV sets (0.75 ± 0.01), and increasing accuracy from the early (0.74 ± 0.01) to mid (0.79 ± 0.01) to late (0.80 ± 0.01) time bins (Fig. 2.4A&B, right panels). We also detected significant interactions between contingency change and time ($F_{(2,100)}=18.86$, $p<0.001$) and set-type and time ($F_{(2,100)}=3.44$, $p<0.05$), indicating significant changes in performance between FAM versus NOV sets between the early to mid time points ($F_{(1,50)}=4.61$, $p<0.05$); this was also true for sets with changed response contingencies from early to mid time points ($F_{(1,50)}=30.98$ $p<0.001$). Furthermore, a significant set-type×contingency change interaction ($F_{(1,50)}=43.97$, $p<0.001$) indicated that performance on FAM sets was higher overall for the set with changed response contingencies (0.81 ± 0.01) relative to the set that did not change (0.78 ± 0.02). In contrast, for NOV sets, overall accuracy was lower for the set with changed response contingencies (0.73 ± 0.01) relative to the set without (0.78 ± 0.02). These findings are consistent with our prior findings with this task (McKim et al., 2016a). We found no significant effect of group ($F_{(1,50)}=0.58$, $p=0.57$) nor a group interaction with set-type, contingency change or time (all F's<1.69, p's>0.16). The absence of group effects indicate that stress did not impact overall accuracy over time following response devaluation.

We also evaluated task performance post-contingency change in terms of RTs, conducting an identical ANOVA to that described above, but taking RT as the dependent measure. We found significant main effects of set-type ($F_{(1,50)}=11.36$, $p=0.001$), with faster RTs for FAM sets (500.15 ± 6ms) relative to NOV sets (509.27 ± 6ms), and contingency change ($F_{(1,50)}=7.23$, $p=0.01$), with slower RTs for sets with changed response contingencies (509.24 ± 6ms) compared to those that did not change (500.18 ± 7ms). A main effect of time ($F_{(1.60, 79.78)}=5.52$, $p=0.01$) also indicated that RTs decreased from the beginning (509.38 ± 6ms) to the end of Part 2 (499.65 ± 7ms, $p=0.02$). A significant interaction between set-type and contingency change ($F_{(1,50)}=15.16$, $p<0.001$)

demonstrated that NOV sets with changed responses resulted in slower RTs (519ms ± 6ms) relative to changed contingency FAM sets (501ms ± 7ms), which was not the case for sets with unchanged contingencies, whether NOV (499ms ± 7ms), or FAM (499ms ± 6ms). We found no significant effect of group ($F_{(1,50)}$=1.61, p=0.21) or group interaction with set-type, contingency change, or time (all F's<0.71, p's>0.54). The absence of effects with group indicate that stress did not impact RTs over time after the contingency change manipulation.

Habitual Responding: Quantifying Perseverative Errors Post-Contingency Change

To quantify the degree to which responses were habitual, we calculated the percentage of perseverative errors relative to total errors following S-R contingency change (McKim et al., 2016a). A set-type by immersion group mixed model ANOVA found a large main effect of set-type on perseverative errors ($F_{(1,50)}$=30.62, p<0.001 η^2=0.33) as well as a set-type by group interaction ($F_{(2,50)}$=5.56, p=0.007, η^2=0.12). There was a trend for a significant main effect of group ($F_{(2,50)}$=2.66, p=0.08, η^2=0.11). To further evaluate the set by group interaction, a follow-up one-way ANOVA for each set-type (FAM and NOV) demonstrated that for the FAM set, groups differed significantly in terms of the proportion of perseverative errors ($F_{(2,52)}$=6.39, p=0.003, η^2=0.80). This result reflects more perseverative errors committed by the group stressed before HABIT Test Part 1 (0.55 ± 0.13), relative to either the group stressed before HABIT Test Part 2 (0.39 ± 0.11, p=0.005) or the no stress control group (0.42 ± 0.18, p=0.026); the latter two groups did not differ significantly from one another (p=1.00). In contrast to these effects for FAM sets, although performance was nearly equivalent for FAM and NOV sets at the end of the HABIT Test Part 1 (Fig 2.5A), we detected no significant group effect on percentage of perseverative errors for the NOV set with changed S-R contingency ($F_{(2,52)}$=0.86, p=0.43). Thus, the SECPT effects on perseverative responding were specific to the highly over-trained S-R actions.

We next assessed whether perseverative errors were stable over time or if they could be overcome during the post-contingency re-learning period (i.e. HABIT Test Part 2). A set-type by time by group ANOVA found significant main effects of set-type ($F_{(1,50)}$=30.58, p<0.001, η^2=0.17) and time ($F_{(2,100)}$=4.60, p<0.05, η^2=0.02), and a significant interaction between set-type and group ($F_{(2,50)}$=5.72, p<0.05, η^2=0.07; Figure 2.5B). To probe the set-type by group interaction, we ran a mixed model ANOVA (time×group) separately for FAM and NOV set performance. For FAM S-R sets, there was no effect of time on percentage of perseverative errors ($F_{(2,100)}$=2.11, p=0.13); however, we did find a significant effect of immersion group ($F_{(2,50)}$=6.39, p=0.03, η^2=0.26), indicating that the greater tendency of group stressed before HABIT Test Part 1 to commit perseverative errors for the FAM sets that was sustained throughout Part 2 (Fig. 2.5B, dashed line). In contrast, for NOV S-R sets, we detected a significant main effect of time on percentage of perseverative errors ($F_{(2,100)}$=3.89, p=0.02, η^2=0.07), but no main effect of group ($F_{(2,50)}$=0.92, p=0.41), reflecting a significant decrease in perseverative errors from the early (0.35 ± 0.02) to late time point (0.31 ± 0.02, p=0.035) of HABIT Test Part 2 (Fig, 2.5B). These results suggest a more pronounced change in perseverative errors over time for FAM sets relative to Nov S-R sets.

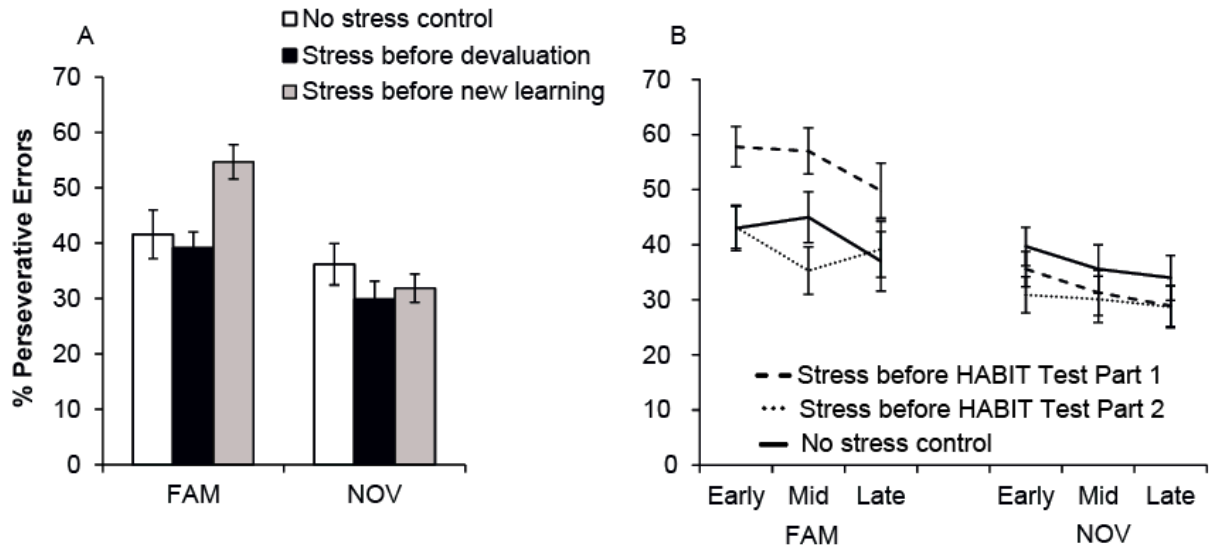

Figure 2.5. Total percentage of perseverative errors and change in perseverative error rate over time by group. (A) We found a significant interaction between set-type and group ($F_{(2,50)}$=5.56, p=0.007, η^2=0.12) that reflected increased perseverative errors for the FAM set ($F_{(2,52)}$=6.39, p=0.003, η^2=0.80). For the FAM set, the group stressed before HABIT Test Part 1 showed a higher proportion of perseverative errors (grey bar; 0.55 ± 0.13) relative to either the group stressed before HABIT Test Part 2 (black bar; 0.39 ± 0.11, p=0.005) or the no stress control group (white bar; 0.42 ± 0.18, p=0.026). There were no significant group effect on percentage of perseverative errors for the NOV set with changed S-R contingency ($F_{(2,52)}$=0.86, p=0.43). (B) We found a significant interaction between set-type and group over time ($F_{(2,50)}$=5.72, p<0.05, η^2=0.07). There was a significant effect of immersion group ($F_{(2,50)}$=6.39, p=0.03, η^2=0.26) for FAM sets, indicating the greater tendency of the group stressed before HABIT Test Part 1 to commit perseverative errors was sustained throughout Part 2. This was not the case for NOV S-R sets, where perseverative errors decreased over time regardless of group ($F_{(2,100)}$=3.89, p=0.02, η^2=0.07).

Independence of Perseverative Error Effects from Cortisol Response

To determine the contribution of cortisol changes to the observed behavioral differences in perseverative errors, we also conducted a two-way ANOVA with immersion group and cortisol responder status as between subject factors on the measurement of perseverative errors for the FAM S-R set with changed response contingencies. This analysis confirmed a main effect of immersion group ($F_{(1,33)}$=12.65, p=0.001), but no main effect of cortisol responder status ($F_{(1,33)}$=0.36,

p=0.55), nor a significant interaction between group and cortisol response ($F_{(1,33)}$=0.007, p=0.93). Thus, the effects of SECPT on habitual responding appears to be independent of an individual's cortisol response to the stressor.

Correlations between Biological Measures of Stress and Perseverative Errors

We examined correlations between the biological measures of stress and perseverative errors for the FAM S-R set with changed response contingencies. We found a trend for a positive correlation between the difference in heart rate from pre- to during stress (Spearman's ρ=0.28, p=0.057); when examining the stress groups combined, this positive correlation was statistically significant (Spearman's ρ=0.40, p=0.02; Fig. 2.6A). In contrast, peak cortisol levels (20 min after stress) did not correlate with perseverative errors in all participants (Spearman's ρ=0.19, p=0.18) or when considering only stressed males (Spearman's ρ=0.05, p=0.79; Fig. 2.6B). We found no significant correlation between IBI variability (lnRMSSD) and perseverative errors when considering all participants (Spearman's ρ=-0.08, p=0.57; Fig. 2.6C), but there was a trend for a negative correlation when considering the stress groups combined (Spearman's ρ=-0.31, p=0.067; Fig. 2.6C). We detected no significant correlations between the HF measure of HRV and perseverative errors (p's>0.16; Fig. 2.6D).

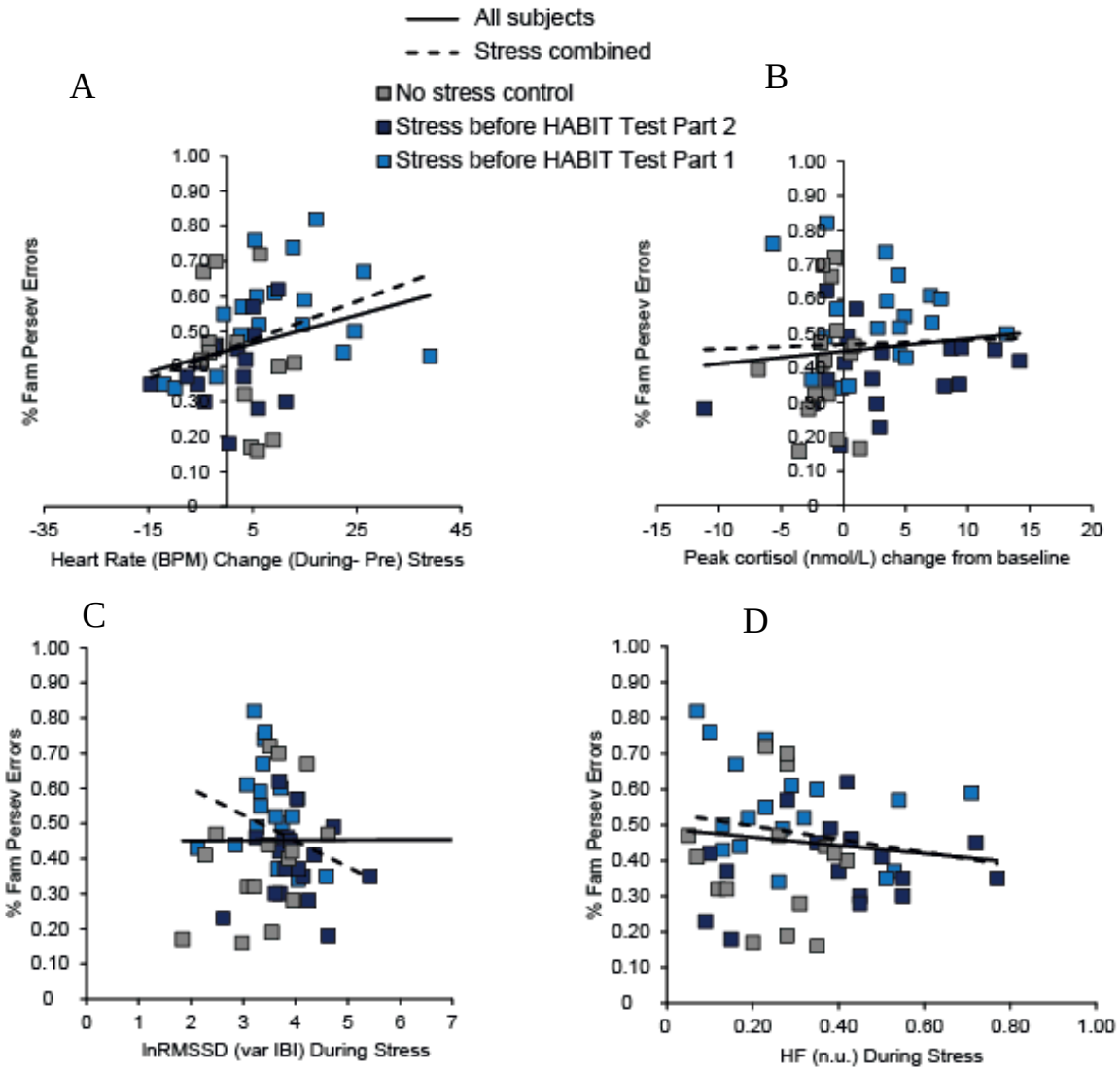

Figure 2.6. Correlations between biological measures of stress and perseverative errors. (A) We found a trend for a positive correlation between the difference in heart rate from pre to during stress (Spearman's ρ=0.28, *p*=0.057). This was significant for the stress groups combined (Spearman's ρ=0.40, *p*=0.02). (B) Peak cortisol levels at 20 min post-stress did not correlate with perseverative errors (*p*'s>0.18). (C) There was no correlation between IBI variability (lnRMSSD) and perseverative errors for all subjects (Spearman's ρ=-0.08, *p*=0.57). A trend was present for the stress groups combined (Spearman's ρ=-0.31, *p*=0.067). (D) There were no significant correlations between HF and perseverative errors (*p*'s>0.16).

Discussion

We investigated the relationship between the timing of acute stress elicited by the SECPT on goal-directed and habitual responding in healthy adult males. We found that stress prior to performance of the HABIT Test Part 1 rendered response behavior habitual during the HABIT Test Part 2; those that experienced the SECPT during this time point in the Test session displayed more perseverative errors relative to both the control group and the group stressed before HABIT Test Part 2, indicating an impairment in overcoming S-R associations for highly over-trained sets. Additionally, we found that this effect was not dependent on cortisol response to the SECPT. For males in the stress before HABIT Test Part 1, there was an increase in perseverative errors regardless of whether their cortisol level rose after the SECPT. This increase in perseverative errors did not occur in individuals stressed before HABIT Test Part 2. Interestingly, those stressed before HABIT Test Part 2 showed higher indices of HRV throughout the Test session, which may have been protective against SECPT effects on perseverative responding.

Our stress timing manipulation facilitated the measurement of acute stress effects of the SECPT on performance of highly practiced S-R sets versus the acquisition of novel S-R associations. We predicted that the FAM S-R sets that were very highly trained and practiced prior to response devaluation become habit-based more rapidly. In contrast, to learn the newly introduced S-R associations, a goal-directed strategy must be utilized, which with time, may switch toward more habit-based response execution; optimal performance during Part 1 of the HABIT task thus relies on both habitual and goal-directed action selection. Our behavioral results demonstrate that task accuracy for FAM sets remains high and stable regardless of an acute stressor, and that the SECPT does not impair learning or acquisition of NOV sets.

Perseverative errors after response devaluation during which S-R contingencies change for FAM and NOV S-R sets are unlikely to reflect generalized deficits in response inhibition based on the following arguments. First, prior to response devaluation, performance on NOV sets was equivalent between stressed and unstressed groups, and deficits in behavior, in the form of perseverative errors, were only evident in the group stressed before HABIT Test Part 1; this result is inline with our previous results in a group of abstinent SUD history individuals, in which perseverative errors were also specific to FAM sets and were not evident for NOV sets with changed contingencies (McKim et al., 2016a). Additionally, further support against the argument of deficits in prefrontal function related to our behavioral findings stem from the lack of evidence in our sample for a relationship between working memory capacity and task performance. Others have demonstrated that working memory capacity is protective against stress effects that shift behavior toward the use of model-free (habit) control of behavior and away from model-based (goal-directed) action selection (Otto et al., 2013). We did not detect differences between groups in working memory capacity (Table 2.1), nor did working memory capacity correlate with HABIT task behavior (data not shown). Moreover, deficits in working memory could also manifest as deficits in task performance after devaluation in terms of either accuracy or perseverative errors for NOV sets, and neither of these effects were detected in our data. Taken together, these ideas lend further support to our interpretation of the stress-induced shift toward more automatic and habitual behavior that is specific to the well-practiced FAM sets.

It is important to note that although the stress groups differed in the timing of the SECPT during the Test session, the groups were matched on subjective ratings of the SECPT and on self-reported daily life (chronic) stress, as well as the duration of hand immersion during the SECPT. This allows us to rule out confounding variables that may have impacted our behavioral results.

However, we did find that males in the stress before Part 2 group showed elevated indices of parasympathetic modulation of heart rate (lnRMSSD and HF). The fact that this group did not increase habitual responding after the SECPT suggests that higher HRV may protect against cognitive impairment effects of stress. An outstanding question is whether the underlying neurobiological correlates or 'state' of continual task performance experienced in Part 1, prior to the stress experience, boosted parasympathetic function that was protective against stress effects of the SECPT in the group stressed before Part 2. It is plausible that cognitive functions necessary for HABIT performance, including sustained attention, working memory, task-switching, and the coordination of motor responding to sensory information, were able to shift the balance of autonomic nervous system control through brain-body interactions. Support for this idea comes from a recent study showing that, in males, changes in psychophysiological responses to stress (measured by SNS activation and alpha EEG oscillation frequencies) were found to modulate individual variability in initial and longer lasting coping responses to stress (Aftanas, 2015). The authors classified individuals based on high or low blood pressure reactivity to stress, which then correlated with differences in theta and alpha frequency oscillations. Specifically, theta band power positively correlated with cardiovascular reactivity (blood pressure and heart rate), posited to represent the magnitude of a bodily response that may be mounted toward the stressor, or an 'aversive motivational' state. In contrast, alpha synchronization of the frontal, frontocentral, central, and centro-parietal scalp electrodes negatively correlated with stress reactivity measures in the low stress response group. At the neural level, activity within these individuals was predicted to returned to a baseline state or even reflect enhanced top-down control and inhibition of subcortical appraisal of threat; however, in males with high stress reactivity, there was weak or absent alpha synchronization that was hypothesized to reflect heightened attention and an inability

to suppress an extended and elevated response to the stressor. Although it is unlikely based on our metrics of the biological stress response that all males within the stress before HABIT Test Part 2 group had 'weak' stress reactivity (notably, more than half were 'cortisol responders'), it is tempting to speculate that the cognitive functions necessary for optimal HABIT performance in Part 1 may have primed and facilitated the suppression of a cardiovascular stress response by synchronization of alpha oscillations among distributed brain networks.

Potential limitations of our study should be noted. First, while we were able to vary the timing of the stress manipulation to test effects on performance of habitual versus acquisition of S-R sets with a goal-directed strategy, as well as the ability to change S-R associations after response devaluation, our group sample sizes are small. This may contribute to the lack of effect of stress on behavior in males stressed before HABIT Test Part 2. However, our sample size had 93% power to detect a medium size group by set-type interaction on perseverative errors, and 96% power to detect a medium sized main effect of set-type on perseverative error within groups. Thus, we can conclude that engaging in the HABIT Part 1 immediately prior to the SECPT protected against the increased habitual responding that occurred when the SECPT was administered prior to Part 1. Additionally, the underlying mechanism of increased HRV during and after a stressor in this group are unknown, and future studies will be needed to determine what is happening at the neural level. Our behavioral findings do not directly address the role of neurotransmitter systems within the brain in contributing to stress effects, behavior results, and their interaction, as we did not measure them or their metabolites; potential use of imaging techniques that include PET or genetic studies can shed light on this issue by determining baseline levels of neurotransmitter binding or enzyme activity that may contribute to individual variability in the response to stress. Study of the neurobiological correlates of the stress-induced increase in perseverative errors that

suggests enhanced striatal control of behavior with stress and over-training is also warranted. Finally, it is possible that our SECPT manipulation, with the component of psychosocial evaluation, may not be as effective in eliciting biological markers of stress changes relative to other laboratory paradigms, such as the TSST (Allen et al., 2014) or Montreal Imaging Stress Task (MIST) (Dedovic et al., 2005). Despite existing validation for the SECPT (Schwabe et al., 2008; Schwabe and Wolf, 2009, 2010), it may be possible to further optimize its effectiveness, for instance by restricting experimenter gender. Moreover, it may be more effective in eliciting a stress response in individuals with specific disorders, such as those with social anxiety, who more readily respond to psychosocial stress.

Our results demonstrate that combined physical and psychosocial stress potentiates habitual responding in healthy adult males who are stressed prior to practice of learned S-R sets. This effect was specific to well-learned S-R actions, and did not occur in the execution S-R sets learned after the stressor. We have also demonstrated that stress occurring prior to acquisition of newly introduced S-R sets does not inhibit S-R learning, which heavily recruits DLPFC (Boettiger and D'Esposito, 2005). Such intact learning suggests that changes at the neural level underlying enhanced habitual responding include a shift toward recruitment of striatal circuitry without deficits in prefrontal control. Our findings are relevant to disorders characterized by dysregulated stress responses, such as post-traumatic stress disorder, and those in which changing behavior is necessary for positive health outcomes, such as SUDs. Stress is an important predictor of treatment outcome for SUDs (Sinha, 2009), and given that SUDs disproportionally affect males (Stephens et al., 2016) an understanding of the types of stress that are relevant to preventing relapse are critical. Our findings further demonstrate the importance of the timing of stress on habitual responding, and suggest novel therapeutic interventions, such as pharmacological compounds to

target SNS activity, or behavioral interventions designed to increase parasympathetic modulation as protective measures of stress-induced effects on behavior.

CHAPTER 3

THE EFFECTS OF STRESS TIMING AND MENSTRUAL CYCLE PHASE ON HABITUAL RESPONDING IN FEMALES

Introduction

Stress is an experience common to most individuals, but the events, perception, and reactions to these stressors vary by individual. The subjective as well as biological reaction to stress can also vary based on sex, due to differences in circulating ovarian hormone levels. In females, the menstrual cycle results in fluctuations of sex steroids, particularly estradiol and progesterone, which impact the physiological and subjective response to stress. These cyclic variations in ovarian hormones thus complicate stress research in females. One approach is to study stress effects on behavior (as in Chapter 2) in females is to test women during the menstrual phase of the cycle, when circulating sex steroid levels are low and relatively static (Abraham et al., 1972). However, some research suggests that the cortisol response to a stressor is comparable to males during the luteal phase of the menstrual cycle (Kirschbaum et al., 1999; Kudielka and Kirschbaum, 2005; Kajantie and Phillips, 2006), when circulating sex steroid levels are relatively elevated and static (Abraham et al., 1972). In women, variability in the response to stress at psychological and physiological levels can further be altered by the use of hormonal contraceptives that regulate hormonal fluctuations (Kajantie and Phillips, 2006). Given these factors that can modify the stress response, the study of acute stress manipulations in females within the laboratory is an important avenue to better understand cycle influences on stress at the behavioral, neuroendocrine, and neurobiological levels. These factors may be especially important in

preventing or treating disorders that commonly affect women, including depression and anxiety (Schiller et al., 2016; WHO, 2016).

Commonly used stress paradigms in the laboratory include physical stress in the CPT, psychosocial threat induced via the TSST, and more recently developed paradigms also include a social evaluative component, such as the MIST or the SECPT. While these tasks have been used in both male and female research samples, compared to data reported from males, the results reported from females have been inconsistent; this may stem from differences in the collection and assessment of SNS activity, HPA axis, and subjective aspects of the stress response. For example, cortisol levels have been shown to be lower in women during the luteal phase, relative to males, after the TSST (Kirschbaum et al., 1999; Stephens et al., 2016). However, another study demonstrated no differences in levels of salivary or serum cortisol in response to the TSST between women in the follicular or luteal phases relative to each other or relative to men (Childs et al., 2010). Childs et al. (2010) also showed increased subjective ratings of stress for women relative to men, which was most pronounced during the luteal phase. In contrast, another study employing the modified TSST found that women in the follicular phase demonstrated an inverse relationship between cortisol levels and subjective stress (Duchesne and Pruessner, 2013). Subjective stress findings from female samples using the MIST demonstrate that higher estradiol levels (early versus late follicular phase) are associated with lower levels of distress after the MIST, with no differences in cortisol response to the MIST (Albert et al., 2015). Furthermore, when examining the relationship between different physiological indicators of stress, studies assessing the impact of cycle phase and sympathetic parameters such as heart rate have found no differences between the early follicular phase and ovulation window in terms of cortisol and heart rate (Pico-Alfonso et al., 2007), while others have demonstrated that women are more likely to show higher

parasympathetic modulation of cardiac reactivity assessed by heart rate variability measures at rest (Kajantie and Phillips, 2006). The findings in female studies are also confounded by use of hormonal birth control, which has been found to blunt the cortisol response to acute stress (Kirschbaum et al., 1999; Kajantie and Phillips, 2006). Together, these studies suggest substantial differences between men and women dependent upon the measures used to assess reactions to stress. Furthermore, within women, differences in subjective as well as biological measurements may stem from fluctuations in hormone levels in the regular cyclic state, as well as with the use of hormonal contraceptives.

Variability in the stress response further results from the action of stress and ovarian hormones as potent neuromodulators within the brain. The combination of these effects in the periphery and the brain may have synergistic effects on cognition and behavior. Acute stress is known to increase levels of catecholamines within the brain, particularly within the PFC (Arnsten, 2009, 2015). This brain area is important for working memory, which is defined as the ability to maintain and manipulate or update information during a short period of time (Fuster, 2008). Working memory function has been related to catecholamine levels in an inverted-U-shaped model; optimal performance is achieved with moderate levels of PFC catecholamines, but with decrements in cognition that manifest through behavioral deficits on either side of the curve with too little or too high levels of PFC catecholamines (Cools and D'Esposito, 2011). This same relationship has also been posited with stress in terms of arousal; arousal levels enhance or inhibit performance in an inverted-U-shaped manner (Yerkes and Dodson, 1908; Arnsten, 2009). Taken together, stress may be a way to enhance or decrease behavioral performance on tasks involving working memory to assess PFC function.

To assess changes in hormonal fluctuations within the brain, EEG oscillations and resting

state blood oxygen level dependent (BOLD) activity at rest have been shown to depend on estradiol in free-cycling women and those using hormonal contraceptives (Brotzner et al., 2014; Petersen et al., 2014); there have also been mixed findings on increases in working memory performance during high estradiol phases of the cycle (Rosenberg and Park, 2002; Joseph et al., 2012) but decrements associated with high estradiol levels in the late follicular (Gasbarri et al., 2008) and mid-luteal phase (Man et al., 1999; Schmitt et al., 2005). Although these studies suggest correlations between estradiol and working memory performance, differences could result from the cycle phase window studied, validation of estradiol and progesterone levels, the assessment of subjective stress, or potentially autonomic indicators such as cardiac activity. This suggests the need to assess multiple aspects of the stress response given their interrelationships and demonstrated by the inconsistencies above (Jacobs and D'Esposito, 2011; Smith et al., 2014).

To begin to examine the relationship between psychosocial stress and menstrual cycle phase, we studied goal-directed and habit-based behaviors, which rely on frontostriatal circuitry, and have been shown to be affected by stress at the behavioral and neurobiological level in both males and females. Importantly, previous studies that have examined these behaviors have either focused solely on males (Schwabe et al., 2008), or included females without examining the contribution of cycle phase or hormonal birth control use (Schwabe and Wolf, 2009, 2010; Schwabe et al., 2011b; Schwabe et al., 2012). Additionally, studies utilizing pharmacological manipulations to induce or block stress effects to examine neurobiological changes failed to find evidence for changes at the neural level in the striatum that is typically associated with habit-based responding to devalued outcomes (Schwabe et al., 2011b; Schwabe et al., 2012). It is possible that the lack of evidence for this finding of the neural signature of habitual responding could have been obscured by synergistic effects of ovarian cycle hormones at the neural level; current debate in the

literature exists about whether ovarian cycle hormones are protective of stress effects on cognition. The evidence for a shift toward habits based on behavioral measures, in the absence of a neurobiological effect in the striatum, suggests that variability in ovarian hormones in females, combined with the biological sequelae of stress, may have accounted for the resulting changes in activation of the PFC during task performance.

We tested females in either the menses phase (MP; days 1-7) or the luteal phase (LP; days 19-23) to examine the effects of stress timing on goal-directed and habitual behavior in our HABIT task. We used the same experimental design as in Chapter 2 (Figure 2.1), with the added feature of testing two sets of participants across the hand immersion conditions: MP and LP. As for Chapter 2, we measured stress effects induced by the SECPT on performance of learned S-R sets and learning of new S-R sets, as well as on changing well-learned and recently learned S-R associations. We employed a novel response devaluation manipulation, in which select S-R sets have changed response contingencies. Based on behavioral responses to this manipulation, we quantify response strategy based on the type of errors participants commit. Of particular interest are perseverative errors, or the tendency to continue to press the previously correct response button, instead of trying the other remaining buttons on the keypad to learn the new, correct response. We collected both subjective and physiological indicators of stress response to the SECPT, to verify that stress was induced as intended. We predicted that females would show similar biological and subjective responses to males, regardless of cycle phase.

Methods

Participants

Healthy adult females were recruited from the UNC Chapel Hill campus and surrounding community via advertisements. Participants (*n*=95) were aged 18-40 years old with no known history of neurological disorders, no current psychiatric diagnoses or psychoactive drug or medication use (excluding moderate nicotine, alcohol, and caffeine), and an estimated IQ within the normal range (≥80). Participants were asked to refrain from excessive caffeine intake (no more than their self-reported regular amount), and to refrain from physical exercise for 6 hours prior to the Test session. Subjects participated in two sessions each, which both occurred either during the menses phase of their ovarian cycle (MP; cycle days 1-7; *n*=49) or during the (luteal phase (LP; cycle days 19-23; *n* = 46). Three participants were not able to complete both sessions within a single cycle, but returned during the next cycle, within the same phase, to complete the second study session; upon inspection, their data was not qualitatively different from others within their phase and stress group. The menstrual cycle day and hormonal contraceptive use information was based on self-report. Females recruited to participate in the menses phase were excluded if they self-reported hormonal contraceptives that were continuously released, rather than delivered cyclically (e.g. mirena; *n*=2). Each subject provided written informed consent as approved by the UNC Office of Human Research Ethics.

General Procedure

Study sessions and protocol were similar to that described in Chapter 2. Briefly, two study sessions were completed (Figure 2.1) with at least one intervening night's sleep. Subjects were compensated as described in the Chapter 2 study. During the HABIT Training session, participants

completed standard questionnaires (see "Behavioral Inventories"), followed by training on the computerized S-R learning task (see "Behavioral Task"), and the automated OSPAN working memory task (Unsworth et al., 2005). No stress manipulation occurred in the Training session. Learning and habitual responding was then measured in the HABIT Test session. As in Chapter 2, we used a between subjects design, with random assignment to one of three groups for the Test session: *i*) Stress before HABIT Test Part 1; *ii*) Stress before HABIT Test Part 2; *iii*) No stress control. Sessions took place between 12 pm and 5 pm to control for diurnal cortisol variation. The experimental procedure is illustrated in Figure 2.1.

Behavioral Inventories

We administered a number of standard questionnaires to quantify factors that could impact our results, as detailed in Chapter 2. We quantified alcohol use behavior with the Alcohol Use and Disorders Identification test (AUDIT; Saunders et al., 1993) and substance use behavior with the Drug Use Screening Inventory, Domain I (DUSI-I; Tarter, 1990). We calculated density of familial alcohol abuse using the Family Tree Questionnaire (FTQ; Mann et al., 1985). Neuropsychological questionnaires included the Barratt Impulsivity Scale (BIS-11; Barratt, 1994), Rotter's Locus of Control scale (LOC; Rotter, 1966), the State-Trait Anxiety Inventory (STAI; Spielberger, 1985), the Thought Action Fusion scale (TAF; Shafran et al., 1996), the Connors' Adult ADHD Rating Scale (CAARS; Connors, 1997), and the Perceived Stress Scale (Cohen et al., 1983). Education and occupation were quantified with the Barratt Simplified Measure of Social Status (BSMSS) (BSMSS; Barratt, 2006). We estimated IQ with the Shipley Institute of Living Scale (SILS; Zachary, 1991).

Behavioral Task

Briefly, the HABIT is a S-R learning and re-learning task implemented in E-Prime 2.0 (PST Inc., Pittsburgh, PA). Task details were included in Chapters 1 and 2, and have been described previously (Boettiger and D'Esposito, 2005; McKim et al., 2016a). During the Training session, participants learn two sets of S-R rules to a criterion of ≥ 90% accuracy (FAM sets). In the Test session, participants first demonstrate retention of the previously learned (FAM) associations, then the learning task begins (HABIT Test Part 1; Fig. 2.1). In the learning task, blocks of the two FAM sets are interspersed with blocks of two new stimulus sets (NOV sets), to measure new S-R learning, and blocks of a control condition, consisting of novel, unrelated stimuli (No Rule set); blocks included 15 randomly selected stimuli from the relevant set. Following the HABIT Test Part 1, subjects were informed that the correct responses for two sets (one FAM and one NOV) had changed (HABIT Test Part 2; Fig. 2.1). The proportion of perseverative errors following this "response devaluation" can be taken as an index of the degree to which responses are outcome independent (i.e. habit-based), as opposed to outcome-driven (i.e. goal-directed), thus we can quantify habitual responding when attempting to overcome both well-learned (FAMILIAR) and freshly learned (NOVEL) S-R associations.

Stress *Protocol*

Participants in the stress groups (stress before HABIT Test Part 1, *n*=34; stress before HABIT Test Part 2, *n*=33; detailed below) were exposed to the SECPT, described in detail in Chapter 2, and elsewhere (Schwabe et al., 2008). In brief, participants immersed their non-dominant hand (including the wrist) into ice water (32°F) for up to 3 min. Participants were asked to face and look toward two video cameras, and were told the cameras would record their facial

expressions during the immersion procedure, during which an unsociable and unfamiliar experimenter monitored them. Most unsociable experimenters were female (n=50), but males were employed in 13 of the 63 stress sessions. The gender distribution of experimenters did not differ between stress groups $\chi^2_{(1)}$=0.01, p=0.91 or cycle phase, $\chi^2_{(1)}$=0.55, p=0.46.

The SECPT procedure was the same for both stress groups, but the SECPT timing within the Test session varied between stress groups (Figure 2.1). Participants in the control group immersed their hand (including the wrist) for 3 min in warm water (80°F), and were not videotaped or monitored by an unsociable experimenter. Water temperature differed significantly between immersion groups ($F_{(2,93)}$=3776.92, p<0.001, η^2=0.99), with no difference in water temperature between cycle phase groups ($F_{(1,93)}$=0.20, p=0.66), and no group by cycle phase interaction ($F_{(1,2,93)}$=0.17, p=0.84) on water temperature. The group differences in water temperature was based on significantly colder water in both the stress before Part 1 group (31.44 ± 2.23°F) and the stress before Part 2 group (32.31 ± 1.81°F) relative to the control group (79.42 ± 4.44°F; both p<0.001); water temperature did not differ between stress groups (p=0.56). To assess SECPT response, we collected subjective ratings of the SECPT, along with biological measures of heart rate, salivary cortisol, and salivary α-amylase.

Subjective Stress Ratings

Participants completed subjective ratings immediately after the SECPT (or control condition) via a questionnaire rating the stressfulness, unpleasantness, and painfulness of the experience on a scale from "not at all" (0) to "very much" (10) (Schwabe et al., 2008).

Heart Rate

To measures changes in autonomic nervous system function in response to the SECPT, we measured heart rate at three time points during the Test session (Figure 2.1, hearts). Disposable Ag-AgCl foam electrodes (Biopac Systems, Inc; Goleta, CA) were placed on clean, dry skin according to the three lead system: below the right and left collarbone area, and below the left ribcage. Electrode signals were sent via a Bionomadix wireless transmitter to a receiver/amplifier (Biopac Systems, Inc), and collected in Acqknowledge 4.3 (Biopac Systems, Inc) with a sampling rate of 500 Hz.

We collected heart rate at the three time points during the Test session shown in Figure 2.1. For the pre- and post-immersion heart rate measures, we collected 5 min of resting heart rate while the participant sat at a desk in a quiet room. The duration of heart rate data during the SECPT varied (max=3 min); heart rate was recorded for the duration of hand immersion. Electrocardiography (ECG) data was visualized, cleaned for artifacts, and processed offline using Mindware 3.0 HRV software (MindWare Technologies, Ltd; Gahanna, OH) by a research assistant blind to group assignment.

Heart Rate Variability (HRV) Measures

Heart rate is controlled by parasympathetic and sympathetic input, with tonic parasympathetic inhibition dominating at rest (Ernst, 2014). These two components can be distinguished based on the timescale of their respective input. Sympathetic changes occur more slowly, on the order of seconds, while parasympathetic modulation occurs at the millisecond scale. Thus, beat-to-beat changes in heart rate (HRV) represents vagal (parasympathetic) dominance over sympathetic inputs to the heart (Saul, 1990). To assess HRV responses to stress, we used both

time and frequency domain measures, as described in Chapter 2. The time-domain HRV measure is the lnRMSSD or the variability in the IBI. The frequency domain HRV measure is the power within the high frequency band (HF; 0.15-0.4 Hz), which primarily reflects parasympathetically mediated heart input, reported as normalized units: HF/(LF +HF) (Burr, 2007).

Salivary Cortisol

We measured HPA axis reactivity to stress via salivary cortisol as described in Chapter 2. We collected saliva samples (Sardstedt Inc., Newton, NC) at the six time points in Test session shown in Figure 2.1; samples were stored frozen until assayed. Free cortisol concentrations were measured via ELISA (Salimetrics LLC, State College, PA); inter- and intra-assay coefficients of variance were below 14%. All saliva samples within participant were assayed in duplicate on the same plate. We defined "cortisol responders" based on a salivary cortisol concentration at 20 min post-SECPT ≥15% higher than their baseline salivary cortisol level (Miller et al., 2013).

Salivary α-Amylase

While the heart rate data gives a gross index of SNS activation, salivary α-amylase secretion is controlled by direct sympathetic innervation of the salivary glands (Schumacher et al., 2013). Thus, salivary α-amylase concentration provides a more distinct SNS activation measure. To assay sympathetic response to the SECPT, we assayed α-amylase concentration in the saliva samples collected pre- and post-immersion (both 5 and 20 min post). Salivary α-amylase levels were determined using a kinetic enzyme assay protocol (Salimetrics LLC, State College, PA); saliva samples within participant were assayed in duplicate on the same plate. Inter- and intra-

assay coefficients of variance were below 3%.

Data Analysis

Data analyses matched the methods described in Chapter 2. Briefly, our primary index of task performance was accuracy during the HABIT Test session. Accuracy data in Parts 1 and 2 were each binned in three epochs ("early", "mid", and "late") for each Part. When sphericity assumptions were violated for repeated measures ANOVA, we applied a Greenhouse-Geisser correction. We differentiated error types (perseverative button press, other incorrect button press) in Part 2 to uncover response-selection strategies used by participants. We also collected reaction time (RT) data in each trial. We tested for group differences in demographic and psychometric variables with univariate ANOVA including stress group and cycle phase as factors for continuous measures and χ^2 tests for categorical measures. All post-hoc tests were Bonferroni corrected for multiple comparisons. All data analyses were performed in SPSS 22 (IBM) or SAS 9.4 (Cary, NC).

Results

Demographic and Psychometric Data

Examination of baseline stress levels from the perceived stress scale indicated that four individuals had values greater than one and a half times the standard deviation (score >26 out of 30) of females within their assigned stress group; this resulted in omission of four cases within the LP sample; stress before HABIT Test Part 1 (n=3), and no stress control (n=1). These four individuals were excluded from all analyses based on evidence that self-reported stress impacts

response to an acute stressor (Radenbach et al., 2015b). After removal of these four outliers, univariate ANOVA results did not demonstrate significant stress group by cycle phase interactions (nor main effects) for IQ, SES, and ethnicity (Table 3.1) or any other psychometric variables (Table 3.1). We did detect significant main effects of age and education between groups, reflecting the younger age of the stress before HABIT Test Part 1 group, although the cycle phase main effect and interaction between stress and cycle phase was not significant (Table 3.1). We also found differences in self-reported use of hormonal contraceptives between the stress groups, reflecting a lower incidence of hormonal birth control use in the control group, particularly in the LP sample (Table 3.1). To account for these differences, we included age and hormonal contraceptive use as covariates in all analyses.

Table 3.1. Sample Demographics and Psychometric Data

	Group 1 Stress before Part 1		Group 2 Stress before Part 2		Group 3 No stress control		Stress×Cycle $F(1,2,89)$	p-value
	Menses Phase (d1-7)	Luteal Phase (d19-23)	Menses Phase (d1-7)	Luteal Phase (d19-23)	Menses Phase (d1-7)	Luteal Phase (d19-23)		
Demographics	(n=17)	(n=17)	(n=16)	(n=17)	(n=16)	(n=16)		
Age (yrs)	19 ± 1	19 ± 1	22 ± 4	20 ± 2	23 ± 6	19 ± 2	1.92	**0.15**
SILS (calculated) IQ	103 ± 6	105 ± 7	104 ± 7	104 ± 7	105 ± 6	105 ± 6	0.32	0.73
Education (yrs)	13.59 ± 1.00	13.50 ± 0.52	15.31 ± 1.96	14.06 ± 1.25	14.75 ± 1.91	13.50 ± 1.06	1.76	**0.18**
SES	27 ± 12	18 ± 4	18 ± 6	19 ± 7	23 ± 11	22 ± 12	2.11	0.13
Ethnicity(% non- white)	29	7	38	18	31	13		0.43[#]
Birth Control Use	11	10	9	10	7	3		0.02[#]
Substance Use								
AUDIT Total	5 ± 4	6 ± 3	3 ± 3	4 ± 4	5 ± 4	4 ± 3	0.60	0.55
Consumption	3 ± 2	4 ± 2	3 ± 3	3 ± 2	3 ± 3	3 ± 2	0.55	0.58
Dependence	0.24 ± 0.75	0.41 ± 0.87	0.19 ± 0.54	0.29 ± 0.59	0.31 ± 0.60	0.19 ± 0.40	0.33	0.71
Harm	1.12 ± 1.70	1.36 ± 1.45	0.31 ± 0.60	0.76 ± 1.35	1.19 ± 1.72	1.00 ± 1.25	0.43	0.65
DUSI-I (%)	0.12 ± 0.14	0.14 ± 0.14	0.12 ± 0.16	0.12 ± 0.13	0.15 ± 0.19	0.12 ± 0.11	0.24	0.79
FTQ density (%)	0.16 ± 0.21	0.14 ± 0.23	0.16 ± 0.16	0.14 ± 0.19	0.19 ± 0.21	0.09 ± 0.10	0.50	0.61
Psychometric								
Perceived Stress	16 ± 7	18 ± 4	15 ± 6	14 ± 6	14 ± 5	15 ± 5	0.48	0.62
BIS Total	58 ± 8	57 ± 6	54 ± 9	57 ± 8	58 ± 10	58 ± 9	0.53	0.59
Attention	16 ± 5	16 ± 3	14 ± 3	16 ± 3	16 ± 3	17 ± 4	0.58	0.56
Motor	21 ± 3	20 ± 3	21 ± 3	21 ± 3	21 ± 5	21 ± 4	0.54	0.56
Non-planning	21 ± 3	21 ± 3	20 ± 5	20 ± 4	21 ± 5	20 ± 3	0.12	0.89
LOC	11 ± 4	10 ± 4	11 ± 4	11 ± 3	11 ± 2	12 ± 3	0.66	0.52
STAI-State Anxiety	36 ± 8	39 ± 9	34 ± 11	36 ± 12	33 ± 8	36 ± 15	0.01	0.99
STAI-Trait Anxiety	41 ± 9	43 ± 11	41 ± 8	38 ± 12	42 ± 10	38 ± 9	0.47	0.63
TAF Total	17 ± 15	22 ± 11	19 ± 12	19 ± 12	15 ± 12	24 ± 16	0.98	0.38
Moral	15 ± 11	19 ± 10	16 ± 11	16 ± 10	13 ± 11	21 ± 12	0.15	0.32
Self	1.6 ± 2.5	1.8 ± 2.7	2.2 ± 2.9	2.1 ± 2.3	1.4 ± 1.9	1.7 ± 3.0	0.04	0.96
Others	0.94 ± 3.19	1.00 ± 1.71	0.69 ± 2.02	1.23 ± 2.17	0.50 ± 1.37	1.60 ± 3.64	0.34	0.71
Connors ADHD Scale								
DSM Inattention	5.47 ± 5.63	6.36 ± 3.13	4.80 ± 3.23	6.25 ± 4.78	6.86 ± 6.38	7.13 ± 4.50	0.11	0.89
DSM Hyperactivity	7.27 ± 5.26	6.79 ± 4.04	6.40 ± 2.80	7.13 ± 3.14	8.43 ± 4.43	6.73 ± 5.11	0.62	0.54
DSM ADHD	12.73 ± 10.09	13.14 ± 6.54	11.20 ± 4.93	13.38 ± 6.37	15.29 ± 10.07	13.87 ± 8.94	0.37	0.69

Values are reported as mean ± standard deviation. Reported p-values reflect the results of unpaired two-tailed comparison between groups. IQ, Intelligence Quotient; SES, Socioeconomic Status; AUDIT, Alcohol Use Disorders Identification Test; DUSI-I, Drug Use Screening Inventory, Domain I; FTQ, Family Tree Questionnaire; Barratt Impulsivity Scale; LOC, Locus of Control; SILS, Shipley Institute of Living Scale; STAI, State-Trait Anxiety Inventory; TAF, Thought Action Fusion Scale. ADHD, Attention Deficit Hyperactivity Disorder; DSM, Diagnostic and Statistical Manual. **Boldface** indicates group or cycle phase main effects are significant at $p<0.05$. [#]p-value represents result of Fischer's exact test.

Behavioral Performance during HABIT Training

During the Training session, subjects were required to reach a performance criterion of 90% accuracy for each (FAM) set. The order of FAM sets was counterbalanced across participants and set order did not differ between stress groups, $\chi^2_{(2)}=0.29$, $p=0.86$ or cycle phase $\chi^2_{(2)}=0.72$, $p=0.40$. Training to criterion took ~25 min, with no significant differences between groups in the average number of training blocks (of 40 trials) needed to learn the first FAM set, (MP-Stress before HABIT Test Part 1: 4 ± 3 blocks; MP-Stress before HABIT Test Part 2: 4 ± 2 blocks; MP-No stress control: 3 ± 2 blocks; LP-Stress before HABIT Test Part 1: 3 ± 1 blocks; LP-Stress before HABIT Test Part 2: 3 ± 2 blocks; LP-No stress control: 3 ± 1 blocks; all *p*'s>0.15). Learning the associative (S-R) rules for the second FAM set was always more rapid, and the required number of blocks again did not differ between groups (MP-Stress before HABIT Test Part 1: 3 ± 1 blocks; MP-Stress before HABIT Test Part 2: 3 ± 1 blocks; MP-No stress control: 3 ± 2 blocks; LP-Stress before HABIT Test Part 1: 3 ± 2 blocks; LP-Stress before HABIT Test Part 2: 3 ± 1 blocks; LP-No stress control: 2 ± 1 blocks; all *p*'s>0.32). Participants were then required to reach 70% accuracy in a third practice version of the task with intermingled blocks of FAM sets 1 and 2; there were no between group differences in the number of trials to criterion (all *p*'s>0.33). Thus, training performance between groups was equivalent prior to returning for the HABIT Test session. Additionally, the number of days that elapsed between the HABIT Training and Test sessions did not differ between groups (MP-Stress before HABIT Test Part 1: 2 ± 2 days; MP-Stress before HABIT Test Part 2: 6 ± 13 days; MP-No stress control: 3 ± 5 days; LP-Stress before HABIT Test Part 1: 2 ± 1 days; LP-Stress before HABIT Test Part 2: 4 ± 6 days; LP-No stress control: 2 ± 1 days; *p*'s>0.16). Participants demonstrated retention of previously learned FAM sets by again reaching the performance criterion of 70% in the practice version of the task that included blocks

of both FAM sets 1 and 2 at the start of the Test session. There was no between group difference in number of trials to reach this criterion (p's>0.13).

Subjective, Endocrine, and Autonomic Responses to Stress

We measured stress induction via the SECPT using subjective stress ratings, as well as measures of salivary cortisol, salivary α-amylase, heart rate, and heart rate variability.

Subjective Stress Ratings

We observed a significant effect of immersion group on the duration of hand immersion ($F_{(2,93)}$=23.85, p<0.001), reflecting significantly shorter times in both the stress before HABIT Test Part 1 groups (93.78 ± 67.61 s, p<0.001), and the stress before HABIT Test Part 2 groups (104.64 ± 65.35 s, p<0.001) relative to the control groups (180 s). Critically, duration of ice water submersion did not differ between stress groups (p=1.00). Thus, the two stress groups experienced equivalent cold pressor effects. We did, however, observe a significant interaction between immersion group and cycle phase on the duration of hand immersion ($F_{(2,93)}$=4.90, p=010). This interaction reflects the fact that the MP-stress before HABIT Test Part 1 group immersed their hand for less time (72.00 ± 56.45 s) relative to the MP-stress before HABIT Test Part 2 group (122.20 ± 70.34 s), whereas the LP-stress before HABIT Test Part 1 group immersed their hand longer (120.22 ± 72.48 s) than did the LP-stress before HABIT Test Part 2 group (88.11 ± 57.50 s).

Examining the subjective ratings of the SECPT, ANOVA detected significant effects of immersion group on stressfulness ($F_{(2,89)}$=71.25, p<0.001, η^2=0.61), unpleasantness ($F_{(2,89)}$=220.36, p<0.001, η^2=0.83), and painfulness ($F_{(2,89)}$=23.84, p<0.001, η^2=0.78). Post-hoc tests demonstrated that both stress groups found the hand immersion more stressful, unpleasant, and painful than did the control group (Table 3.2). The only dimension on which stress groups significantly differed from each other was on unpleasantness, where the average score in the stress

before HABIT Test Part 2 groups (8.09 ± 1.47) was significantly higher than that of the stress before HABIT Test Part 1 group (7.19 ± 2.09), regardless of cycle phase (p=0.015; Table 3.2).

Table 3.2. Subjective stress rating and SECPT parameters based on stress timing and menstrual cycle phase

	Group 1 Stress before Part 1		Group 2 Stress before Part 2		Group 3 No stress control		*F*(2,89)	*p*-value
	Menses Phase (d1-7) (*n*=17)	Luteal Phase (d19-23) (*n*=17)	Menses Phase (d1-7) (*n*=16)	Luteal Phase (d19-23) (*n*=17)	Menses Phase (d1-7) (*n*=16)	Luteal Phase (d19-23) (*n*=16)		
Subjective Rating								
Unpleasant	7.71 ± 1.57	6.57 ± 2.50	8.06 ± 1.65	8.12 ± 1.32[#]	1.00 ± 0.97	0.33 ± 0.97[#]	220.36	**<0.001**
Painful	7.12 ± 2.03	5.50 ± 2.38	7.31 ± 1.70	7.24 ± 2.02	0.06 ± 0.25	0.07 ± 0.26[#]	171.56	**<0.001**
Stressful	5.47 ± 2.35	4.43 ± 2.41	5.38 ± 2.13	4.65 ± 2.09	0.19 ± 0.54	0.07 ± 0.26[#]	71.25	**<0.001**
SECPT Parameters								
Time in Water (secs)	72.00 ± 56.44	120.22 ± 72.48	122.20 ± 70.33	88.11 ± 57.49	180	180[#]	23.85 [§]	**<0.001**
Water Temperature (°F)	31.35 ± 2.93	31.55 ± 0.88	32.60 ± 1.25	32.03 ± 1.81	79.51 ± 2.60	79.34 ± 4.44[#]	3488.44	**<0.001**

Values are reported as mean ± standard deviation. Reported *p*-values reflect the results of univariate ANOVA with the factors of stress group and cycle phase. The main effect of stress group is reported, with no significant effects of cycle phase. [#]*Indicates significant difference relative to the stress before HABIT Test Part 1 group resulting from Bonferroni corrected post-hoc tests* [§] *Indicates* significant interaction between stress group and cycle phase.

Salivary Cortisol

To quantify the change in salivary cortisol as a result of hand immersion, we first averaged the cortisol concentrations of the two baseline samples (~5 and ~25 min samples obtained after HABIT Test session start; Fig. 2.1), then subtracted this baseline value from the cortisol concentration in post-immersion samples. We detected no significant differences between groups in average baseline salivary cortisol prior to the SECPT/control immersion (all p's>0.13). Repeated measures ANCOVA results demonstrated a significant time by group interaction ($F_{(4.75,206.67)}$=7.25, p<0.001, η^2=0.14), reflecting significant increases in salivary cortisol 20 min after the SECPT for both stress groups (Fig. 3.1). There were no significant effects of age, use of hormonal contraceptives, cycle phase, nor did any of these variables interact with time (all p's>0.38). Follow-up ANCOVAs for each time point demonstrated that cortisol increases were specific to the 20 min post-stress time point based on main effects of group (F's>4.98, p's<0.009). For the stress before HABIT Test Part 1 group, cortisol levels increased significantly (1.68 ± 0.63 nmol/L) relative to the no stress control group (-1.09 ± 0.62 nmol/L, p=0.003) at the 20 min peak time point. Salivary cortisol levels also increased in the stress before HABIT Test Part 2 group (1.27 ± 0.69 nmol/L), relative to the no stress control group (-1.76 ± 0.73 nmol/L, p=0.003) at the 20 min peak. Salivary cortisol levels at the end of the HABIT Test session did not differ between groups, or based on any control variables (p's>0.10). Together, these data indicate that the SECPT effectively induced cortisol release in both stress groups.

In addition to measuring changes in salivary cortisol for all individuals within the sample, following the method of Miller et al. (2013), we also separately evaluated 'stress responders' based on salivary cortisol response (see Methods). The proportion of cortisol responders did not differ between stress groups (stressed before HABIT Test Part 1, n=14/31; stressed before HABIT Test

Part 2, n=18/33; $\chi^2_{(1)}$=0.56, p=0.45), or cycle phase (MP, n=18/33; LP, n=14/31; $\chi^2_{(1)}$=0.56, p=0.45).

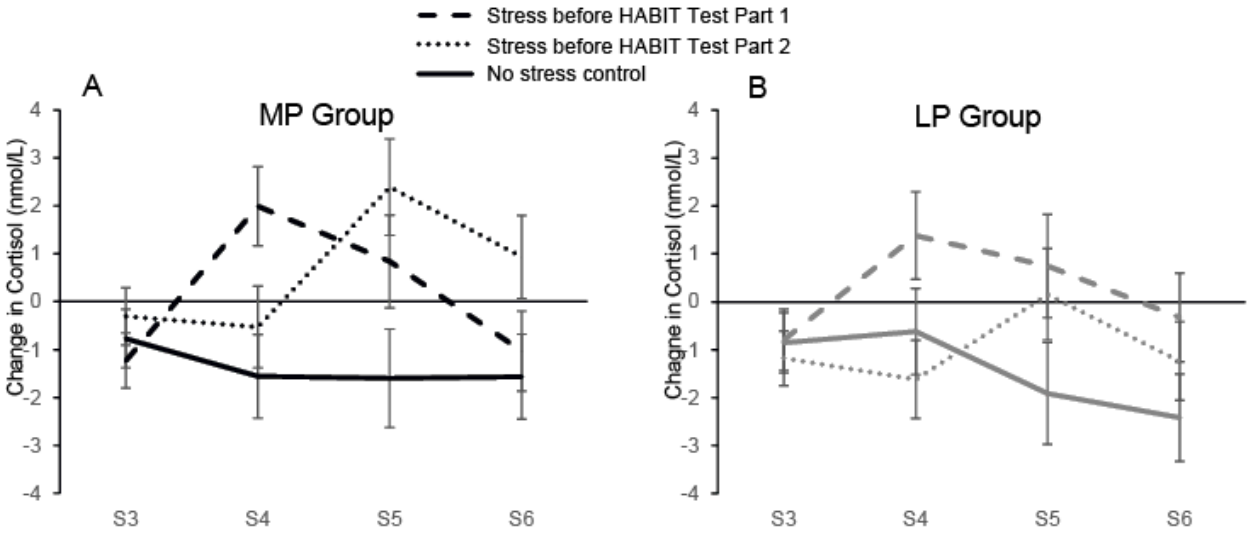

Figure 3.1. Salivary cortisol change over time relative to baseline cortisol average by stress group and cycle phase. Solid lines represent the control group, dashed lines represent the stress before HABIT Test Part 1, and dotted lines represent the stress before HABIT Test Part 2. Plot illustrates the time course of the change in salivary cortisol values (nmol/L) as a function of stress group and (A) menses phase (MP) or (B) luteal phase (LP) group. Significant changes in cortisol (time×group interaction: $F_{(4.75,206.67)}$=7.25, p<0.001, η^2=0.14) measured at 20 min post stress for females stressed before HABIT Test Part 1 (S4; 1.68 ± 0.63 nmol/L) and females stressed before HABIT Test Part 2 (S5; 1.27 ± 0.69 nmol/L) showed significant increases relative to the control group at S4 (-1.09 ± 0.62 nmol/L) and S5 (-1.76 ± 0.73 nmol/L). Cortisol levels at baseline (S3) and at the end of the study session (S6) did not differ between groups (p's>0.10).

Heart Rate Measures

Heart Rate data

Heart rate data was collected a three time points during the Test session to measures changes in stress over time (Fig. 2.1). Results from our study in males using the same study protocol demonstrated increases in heart rate during stress for the group stressed before HABIT Test Part 1, but not males stressed prior to HABIT Test Part 2. Repeated measures ANCOVA to test differences between groups across the Test session in females showed a main effect of time

($F_{(1.57,133.04)}$=7.81, p=0.002, η^2=0.06; Fig. 3.2), with planned comparisons demonstrating that heart rate increased from before to during immersion ($F_{(1,85)}$=6.82, p=0.01) and significantly decreased from during to ~5 minutes post-immersion ($F_{(1,85)}$=11.15, p=0.001; Figure 3.2, top panels). We also found a small time by cycle phase interaction ($F_{(1.57,133.04)}$=5.34, p=0.010, η^2=0.04), due to a larger increase in heart rate during immersion (84.97 ± 2.10 BPM), relative to baseline (76.17 ± 1.74 BPM), in the MP group, relative to the LP group (baseline: 77.30 ± 1.84 BPM; during immersion: 79.42 ± 2.23 BPM). More importantly, there was a substantial main effect of immersion group ($F_{(2,85)}$=6.00, p=0.004, η^2=0.11), as well as a time by immersion group interaction ($F_{(3.13,,133.04)}$=9.08, p<0.001, η^2=0.12; Fig. 3.2.). We also found a substantial main effect of group on heart rate during immersion ($F_{(2,85)}$=11.45, p<0.001, η^2=0.20), demonstrating that females in the stress before HABIT Test Part 2 group (90.62 ± 2.56 BPM; p<0.001) and females in the stress before HABIT Test Part 1 (83.06 ± 2.76 BPM; p=0.012) had higher heart rate during hand immersion compared to the no stress control group (72.89 ± 2.67 BPM). After stress, there was no significant between group effects ($F_{(2,87)}$=1.77, p=0.18) and no effects of cycle phase or other variables or their interaction (all p's>0.11), suggesting a return to similar heart rate levels. We did find a main effect of group on baseline heart rate ($F_{(2,87)}$=3.69, p=0.029, η^2=0.07), driven by higher baseline heart rate in the stress before HABIT Test Part 2 group (81.44 ± 2.07) relative to the no stress control group (73.91 ± 2.20), which may have contributed to the smaller changes in heart rate that occurred during stress in the LP female group relative to the MP female group. In contrast to male heart rate data, both female stress groups showed increased heart rate during stress, whereas only males that were stressed prior to HABIT Test Part 1 demonstrated significant increases during immersion. We also found that females in the LP group showed smaller increases in heart rate from baseline to immersion relative to the MP group.

Heart rate variability (HRV) measure I: RMSSD

In an effort to isolate changes in parasympathetic activity during the Test session, we also examined HRV. To do so, we first quantified variance in the IBI of successive heart beats as the lnRMSSD (see Methods). In males, we found higher measures of HRV in the group stressed before HABIT Test Part 2 relative to both the control group and the stress before HABIT Test Part 1 group. In contrast, the group stressed prior to HABIT Test Part 1 showed decreases in the variability of the IBI, indicating a relative dominance of sympathetic activity during stress. A repeated measures ANCOVA for our female data showed a main effect of time ($F_{(1.67,142.06)}$=6.84, p=0.003 η^2=0.06), demonstrated by a trend for a decrease in the variability of the lnRMSSD during immersion relative to baseline ($F_{(1,85)}$=3.24, p=0.075) and a significant increase in lnRMSSD variability post-immersion ($F_{(1,85)}$=11.48, p=0.001; Fig. 3.2). There was also a significant interaction between time and group ($F_{(3.34,142.06)}$=3.21, p=0.021, η^2=0.06). Follow-up ANCOVA showed no significant difference between immersion groups in lnRMSSD prior to (all p's>0.12) or after immersion (all p's>0.09); however, a significant difference in lnRMSSD between immersion groups occurred during immersion ($F_{(2,85)}$=3.94, p=0.023, η^2=0.08; Figure 3.2, lower panels); this finding reflects lower variability in the lnRMSSD in both the stress before HABIT Test Part 1 group (3.72 ± 0.17) and the stress before HABIT Test Part 2 group (3.35 ± 0.15) relative to the control group (3.97 ± 0.16). We found no effects of lnRMSSD variability over time between cycle groups ($F_{(1.67,142.06)}$=1.60, p=0.21). Results from females demonstrate that variability in the IBI decreases during stress and that both stress groups showed decreases in this index during immersion, with no effects of cycle phase on this measure of HRV.

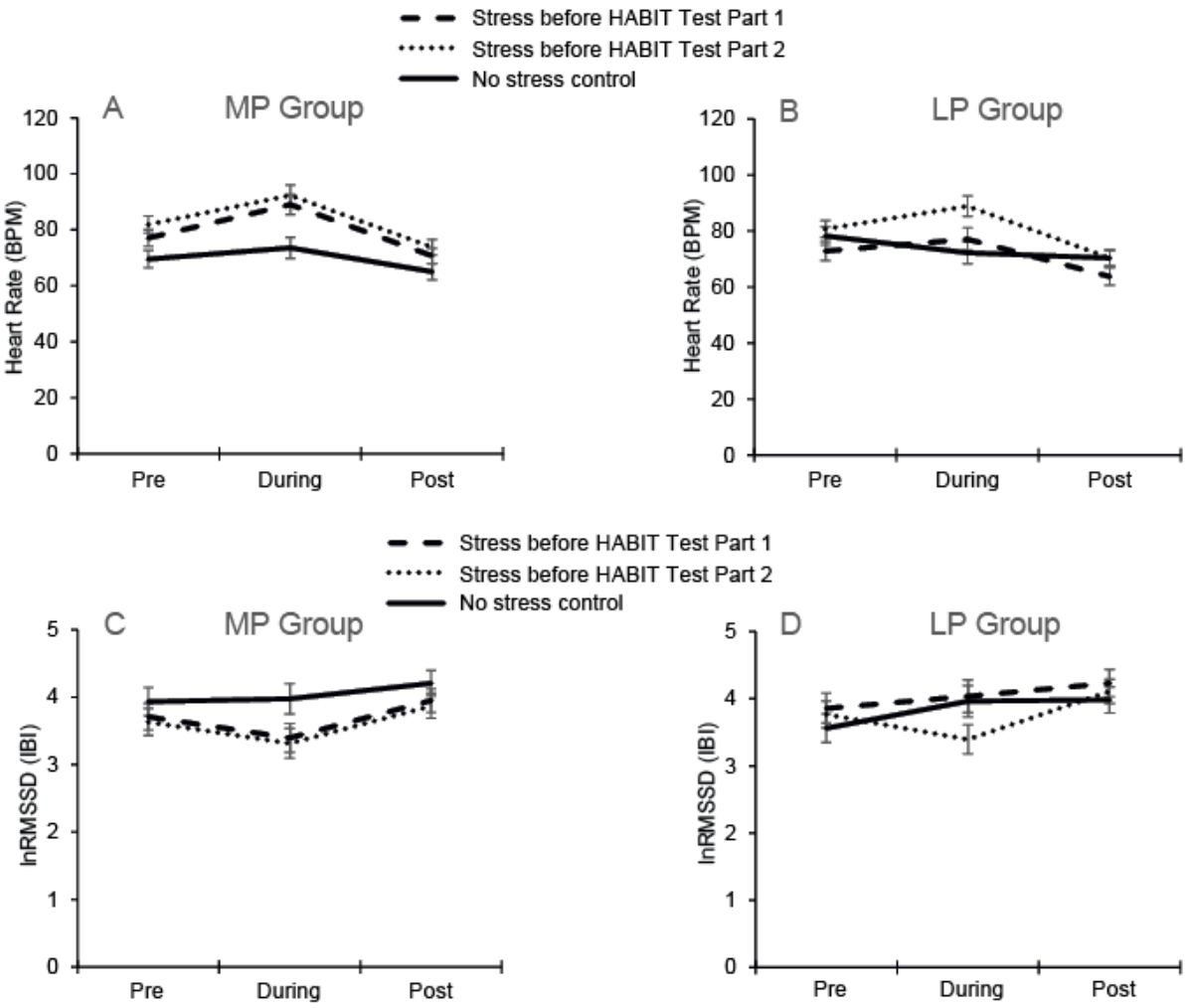

Figure 3.2. Heart rate change over time and lnRMSSD by stress group and cycle phase. Solid lines represent the control group, dashed lines represent the stress before HABIT Test Part 1, and dotted lines represent the stress before HABIT Test Part 2. Plot illustrates the time course of the change in heart rate (A, top; BPM) and heart rate variability (B, bottom; interbeat interval (IBI)) as an index of parasympathetic activity by stress group and (left column: A&C) menses phase (MP) or (right column B&D) luteal phase (LP) group. Top panels (A&B): We found a significant time by cycle phase interaction ($F_{(1.57,133.04)}$=5.34, p=0.010, η^2=0.04), such that the MP group showed a larger increase from pre-stress HR (76.17 ± 1.74 BPM) to HR during immersion (84.97 ± 2.10 BPM), (B) while the change was smaller in the LP group from pre- (77.30 ± 1.84 BPM) to during immersion (79.42 ± 2.23 BPM). A time by group interaction ($F_{(3.13,,133.04)}$=9.08, p<0.001, η^2=0.12) demonstrated that females in the stress before HABIT Test Part 2 group (90.62 ± 2.56 BPM; p<0.001) and females in the stress before HABIT Test Part 1 (83.06 ± 2.76 BPM; p=0.012) had higher HR during stress compared to the no stress control group (72.89 ± 2.67 BPM). Bottom panels (C&D): Significant effects of time ($F_{(1.67,142.06)}$=6.84, p=0.003 η^2=0.06) and a time×group interaction ($F_{(3.34,142.06)}$=3.21, p=0.021, η^2=0.06) demonstrated differences in IBI during immersion (F(2,85)=3.94, p=0.023, η2=0.08). The stress before HABIT Test Part 1 group (3.72 ± 0.17) and the stress before HABIT Test Part 2 group (3.35 ± 0.15) had lower IBI's relative to the control group (3.97 ± 0.16), indicating faster heart rate during immersion.

Heart rate variability (HRV) measure II: HF

In addition to evaluating HRV with a time domain measure, we also used a frequency domain analysis, focusing on power in the HF component of the power spectrum (see Methods), which is thought to reflect parasympathetic activity. A repeated measure ANCOVA detected no significant effect of time ($F_{(1.79,150.54)}$=0.99, p=0.37), nor a time by immersion group interaction ($F_{(3.58,150.54)}$=0.18, p=0.95) on HF power. There were also no differences in this measure over time between cycle phase groups ($F_{(1.79,150.54)}$=2.20, p=0.12), and furthermore, no effects or interactions with covariates within the model (all p's>0.09).

Salivary α-Amylase

As a means of assessing sympathetic activation, we analyzed changes in salivary α-amylase at 5 min and 20 min post-immersion, anticipating a rise in salivary α-amylase at 5 min in the stressed groups, with a return to baseline values after the 20 min post-SECPT measurement. However, a repeated measure ANOVA detected a trend for a main effect of time ($F_{(2,174)}$=2.62, p=0.076, η^2=0.03), but no effect of immersion group ($F_{(2,87)}$=2.02, p=0.14, η^2=0.04), and no interaction between time and immersion group ($F_{(4,174)}$=1.15, p=0.34 η^2=0.02). We found no effects or interactions with cycle phase (F's<1.06, p's>0.038).

Behavioral Results

Stress Effects on Learning New Sets and Execution of Familiar S-R Sets

We next tested the impact of our stress manipulation on behavior. We previously found that males stressed prior to HABIT Test Part 1 performed slighty better at practiced sets, but were not impaired in learning new S-R associations after stress. To assess performance pre-contingency change in females, we conducted a mixed model repeated measures ANCOVA with set-type (FAM/NOV) and time (early, mid, late) as within subject factors, and stress group, coded as participants stressed prior to HABIT Test Part 1 (n=30) versus not (n=65), and cycle phase as between subjects factors. We found an expected main effect of time ($F_{(1.87,166.08)}$=4.69, p=0.010, η^2=0.01), with accuracy improving from early (0.65 ± 0.01) to mid (0.75 ± 0.01) to late (0.78 ± 0.01) runs (Fig. 3.3). Consistent with previous studies with this task (Boettiger and D'Esposito, 2005; McKim et al., 2016a; Chapter 2), we found a set-type by time interaction ($F_{(1.85,166.08)}$=3.89, p=0.026, η^2=0.01), reflecting a greater improvement of NOV set performance over time (Fig. 3.3). To decompose the significant interaction between set-type and time, we ran separate repeated measures ANCOVAs to evaluate time and group effects on accuracy for each set-type. For the FAM sets, there were no significant effects of time ($F_{(2,178)=}$0.63, p=0.53) nor an interaction between time and group ($F_{(2,178)=}$0.16, p=0.86); thus, accuracy was static during the HABIT Test Part 1 for well-practiced sets, regardless of acute stress. We detected no significant effects of cycle phase nor effects of covariates (age, hormonal contraceptive use; all p's>0.07). In contrast, for the NOV sets, there was a significant main effect of time ($F_{(1.85,164.32)}$=6.17, p=0.003, η^2=0.06), and no interaction between time and stress/no stress group ($F_{(1.84,164.32)}$=0.79, p=0.46). Contrasts revealed that accuracy dramatically improved from early to mid Part 1 ($F_{(1,89)}$=9.46, p<0.001), but not from mid to late Part 1 ($F_{(1,89)}$=0.09, p=0.77). There was also a main effect of cycle phase ($F_{(1,89)}$=4.42, p=0.038, η^2=0.04), driven by females in the LP group showing higher accuracy (0.69 ± 0.02)

overall for NOV sets relative to females in the MP group (0.65 ± 0.01). We also detected a main effect of hormonal contraceptive use ($F_{(1,89)}$=4.28, p=0.042, η^2=0.04), reflecting greater accuracy for females taking hormonal contraceptives compared to females not taking hormonal contraceptives; hormonal contraceptive use did not interact with other factors (all p's>0.16). The latter two findings suggest that higher levels of ovarian hormones, whether endogenous (LP) or exogenous (hormonal birth control) improve new S-R acquisition.

We further assessed changes in performance in terms of RTs, conducting an identical ANCOVA to that described above, but taking RT as the dependent measure. No significant main effects of time ($F_{(1.64,146.07)}$=0.07, p=0.91), set-type ($F_{(1,89)}$=0.61, p=0.44), or their interaction with group (p's>0.15) were found; there were also no effects of covariates (all p's>0.25). Interestingly, there was a significant main effect of group ($F_{(1,89)}$=4.16, p=0.04, η^2=0.04), based on females experiencing the SECPT before Part 1 showing faster RTs overall (495.88 ± 7.17ms) compared to females that had <u>not</u> experienced the SECPT before Part 1 (513.83 ± 4.77ms).

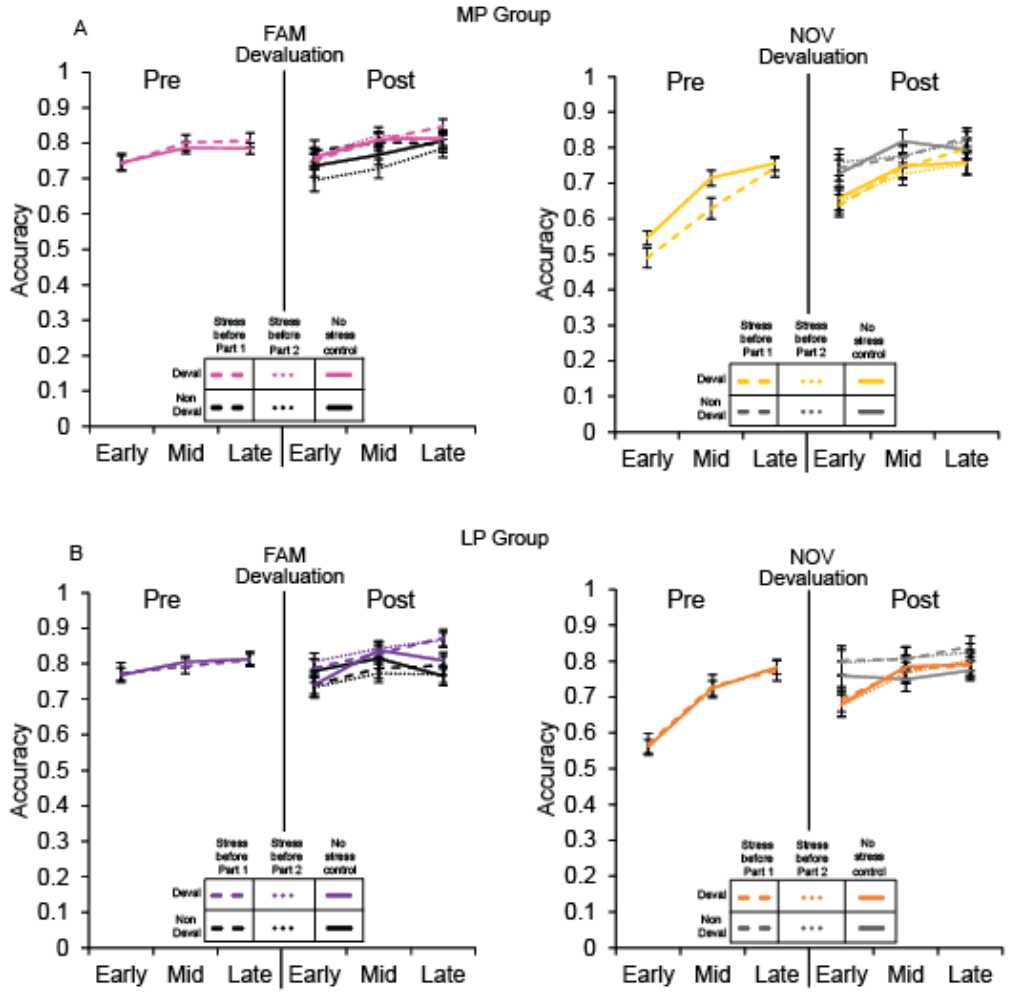

Figure 3.3. Accuracy performance for FAM and NOV sets before and after response devaluation by stress group and cycle phase. Solid lines represent the control group, dashed lines represent the stress before HABIT Test Part 1, and dotted lines represent the stress before HABIT Test Part 2. Female MP cycle group is displayed on the top panel (A) and LP cycle group is displayed in the bottom panel (B). For accuracy prior to devaluation (left panels), we found a set-type by time interaction ($F_{(1.85,166.08)}$=3.89, p=0.026, η^2=0.01), reflecting a greater improvement of NOV set performance over time. For FAM sets, there were no significant effects of time ($F_{(2,178)}$=0.63, p=0.53) nor an interaction between time and group ($F_{(2,178)}$=0.16, p=0.86). In contrast, NOV sets showed a significant effect of time ($F_{(1.85,164.32)}$=6.17, p=0.003, η^2=0.06), and no interaction between time and group ($F_{(1.84,164.32)}$=0.79, p=0.46). There was also a main effect of cycle phase ($F_{(1,89)}$=4.42, p=0.038, η^2=0.04), driven by females in the LP group showing higher accuracy (0.69 ± 0.02) overall for NOV sets relative to females in the MP group (0.65 ± 0.01). Accuracy performance post-devaluation (right panels) demonstrated a significant interaction between set-type and time interaction ($F_{(1.93,168.03)}$=5.39, p=0.006, η^2=0.004), and a four way interaction between set-type, time, group and cycle phase ($F_{(1.59,1.93,3.86,168.03)}$=2.53, p=0.044, η^2=0.004). Follow-up analyses showed that there was a marginal interaction between contingency change, time, and group ($F_{(3.65,166.15)}$=2.33, p=0.074, η^2=0.01) for FAM sets, demonstrating larger changes in accuracy over time for changed contingency sets in females that were stressed prior to HABIT Test Part 1.

Behavioral Performance in Part 2 for Familiar and Novel S-R Sets

We also tested the impact of stress preceding our response devaluation manipulation, to determine whether stress impairs the ability to overcome over-trained S-R associations. In males, we saw deficits that were specific to perseverative responding in the group stressed prior to HABIT Test Part 1, as opposed to those stressed before behavior measured in HABIT Test Part 2. For accuracy post-contingency change in females, we first conducted a mixed model ANCOVA with within subject factors of set-type (FAM or NOV set), contingency change (yes or no), and time (early, mid, late), with immersion group and cycle phase as a between subjects factors. In contrast with our earlier findings with males (Chapter 2), we found no significant effects of time ($F_{(1.59,138.11)}$=0.37, p=0.69), set-type ($F_{(1,87)}$=0.05, p=0.83), or their interaction with immersion group ($F_{(1.59,1.93,,168.03)}$=0.06, p=0.99). In contrast, we found a very small set-type by time interaction ($F_{(1.93,168.03)}$=5.39, p=0.006, η^2=0.004; Fig. 3.3), and another small interaction between set-type, time, and age ($F_{(1.93,168.03)}$=6.21, p=0.003, η^2=0.004); older individuals were more likely to show lower accuracy values relative to younger individuals; age did not interact with other variables (all p's>0.33). We also found a significant four way interaction between set-type, time, group and cycle phase ($F_{(1.59,1.93,3.86,168.03)}$=2.53, p=0.044, η^2=0.004). To decompose these effects, we ran separate mixed model ANCOVAs for each set-type. For FAM sets, we found no main effect of contingency change ($F_{(1,91)}$=1.34, p=0.25), and a small marginal effect of time ($F_{(1.78,162.15)}$=2.68, p=0.078, η^2=0.01). We also found a small interaction between age and time ($F_{(1.78,162.15)}$=3.40, p=0.04, η^2=0.01), suggesting that older individuals had larger improvements in accuracy over time post-contingency change. There was additionally a small, marginal interaction between contingency change, time, and group ($F_{(3.65,166.15)}$=2.33, p=0.074, η^2=0.01; Fig. 3.3), demonstrating larger changes in accuracy over time for changed contingency sets in females that were stressed prior to Part 1. For NOV sets, there were no main effects of contingency change, time, nor their

interaction (F's<2.31, p's>0.10). We did find a significant main effect of hormonal contraceptive use ($F_{(1,91)}$=5.91, p=0.02, η^2=0.06), suggesting that females taking hormonal contraceptives showed higher accuracy for NOV S-R sets overall compared to females not taking hormonal contraceptives, similar to our findings from the HABIT Test Part 1. We additionally found no interacting effects of immersion group (F's<1.22, p's>0.31). The absence of effects with group indicate that stress did not significantly impact performance over time in accuracy after response devaluation.

We also evaluated task performance post-contingency change in terms of RTs, conducting an identical ANCOVA to that described above, but taking RT as the dependent measure. We found trends for effects of response devaluation on RTs, such that RT was slowed for sets with changed contingencies ($F_{(1,87)}$=3.62, p=0.06, η^2=0.005). There was also a trend for an interaction between contingency change and immersion group ($F_{(2,87)}$=3.05, p=0.052, η^2=0.009), further demonstrating slowed RTs for the stress groups relative to the non-stressed control group. We found that cycle phase interacted with set-type ($F_{(1,87)}$=5.15, p=0.03, η^2=0.009), resulting in slower RTs for females in the LP group for FAM and NOV sets relative to females in the MP. We additionally found higher order interactions of set-type, time, group and cycle phase ($F_{(1.93,\ 3.85,167.55)}$=3.06, p=0.020, η^2=0.008) and contingency change with time, group, and cycle phase ($F_{(1.93,\ 3.68,167.55)}$=3.36, p=0.014, η^2=0.005). To decompose these effects, we ran separate mixed model ANCOVAs for each set-type. For FAM S-R sets, we found a significant interaction between time and group ($F_{(3.69,167.93)}$=2.58, p=0.044, η^2=0.02), which was driven by a decrease in RT in the control group relative to the stress groups over time. We also found a small interaction between the covariate of age and contingency change ($F_{(1,91)}$=7.38, p=0.008, η^2=0.03), suggesting that older individuals showed slower RTs for non-changed contingency sets relative to S-R sets with changed

contingencies. There was also a significant four-way interaction between contingency change, time, group, and cycle phase ($F_{(1.00,1.84,3.88,176.56)}$=3.32, p=0.012, η^2=0.02). For females in the LP group, RT decreased for changed contingency sets relative to non-changed contingency sets for the control and stress before HABIT Test Part 2 group relative to the stress before HABIT Test Part 1 group. In contrast, females in the MP group showed increased RTs for the changed contingency sets relative to non-changed contingency sets for both stress groups relative to the control group. For NOV S-R sets, we found an interaction between contingency change, time, and cycle phase ($F_{(1,2,182)}$=5.33, p=0.006, η^2=0.008), which appears to be driven by larger changes in RTs for females in the MP group, with RTs decreasing for changed contingency sets while RTs increased for non-changed S-R contingency sets. There were no effects of group or their interaction for NOV sets, which mirrored the accuracy findings, and suggest that group effects were evident for RTs in FAM sets relative to NOV sets.

Habitual Responding: Quantifying Perseverative Errors Post-Contingency Change

To examine the habitual nature of responding, we tested the difference between groups in the percentage of perseverative errors relative to total errors post-contingency change of the changed NOV and FAM sets (McKim et al., 2016a). A mixed model ANCOVA with set-type as the within subjects factor showed no significant effects of set-type on the overall percentage of perseverative errors ($F_{(1,87)}$=1.83, p=0.18) nor a set-type by group interaction ($F_{(2,87)}$=0.52, p=0.60; Fig. 3.4). There were also no other significant interactions or main effects (p's>0.18).

We next assessed whether perseverative errors were likely to increase initially after devaluation or whether they remained stable over time. We again found no effect of set-type on the overall percentage of perseverative errors ($F_{(1,87)}$=1.69, p=0.20), time ($F_{(1,87)}$=1.39, p=0.25), or higher order interaction of set-type by group by time ($F_{(1,4,174)}$=1.71, p=0.15; Fig. 3.4). There were

also no other significant interactions or main effects (*p*'s>0.09). SECPT timing did not result in effects on perseverative responding, in contrast to what we previously demonstrated in healthy males (Chapter 2).

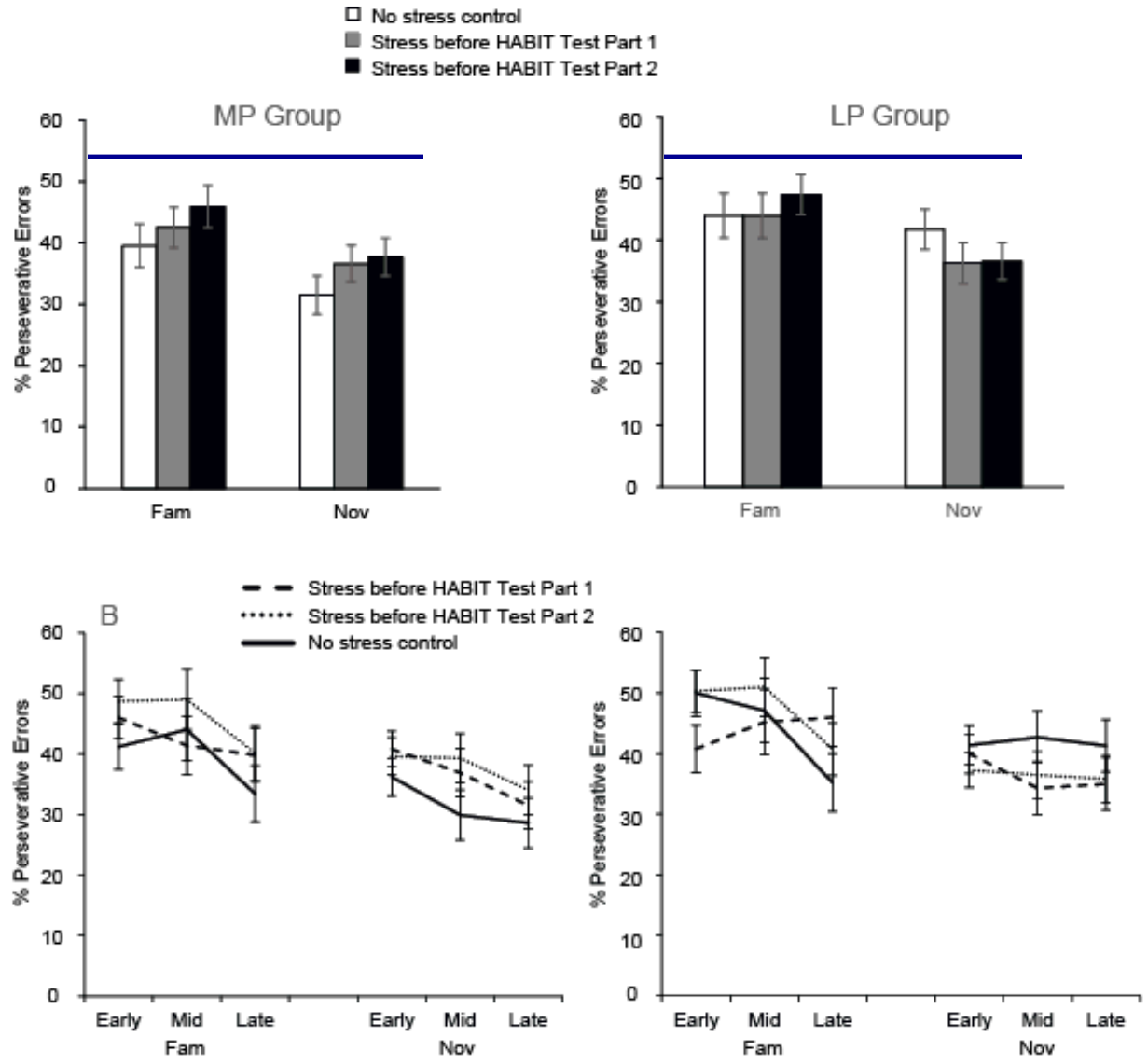

Figure 3.4. Total percentage of perseverative errors for FAM and NOV sets (top) and change over time (bottom) by group and cycle phase. panel: Open bars represent the control group, gray bars represent the stress before HABIT Test Part 1, and black bars represent the stress before HABIT Test Part 2. Bottom panel: Solid lines represent the control group, dashed lines represent the stress before HABIT Test Part 1, and dotted lines represent the stress before HABIT Test Part 2. Top panel: We found no significant effects on the overall percentage of perseverative errors ($F_{(1,87)}$=1.83, p=0.18) nor a set-type by group interaction ($F_{(2,87)}$=0.52, p=0.60). Bottom panel: We also found no main effect of set-type ($F_{(1,87)}$=1.69, p=0.20), time ($F_{(1,87)}$=1.39, p=0.25), or higher order interaction of set-type×group×time ($F_{(1,4,174)}$=1.71, p=0.15). Solid blue line denotes mean perseverative responses for males stressed before HABIT Test Part 1.

Independence of Perseverative Error Effects from Cortisol Response

To determine the contribution of cortisol changes to individual variability in perseverative errors, we also conducted an ANCOVA with immersion group, cycle phase, and cortisol responder status as between subject factors on the measurement of perseverative errors for the FAM S-R set with changed response contingencies. This analysis demonstrated no significant main or interacting effects of cortisol responder status (F's<1.92, p's>0.17), nor any other variables (F's<3.46, p's>0.07). Thus, the lack of SECPT effect on perseverative errors does not appear to reflect a deficit in cortisol signaling in response to the stress manipulation.

Correlations between Biological Measures of Stress and Perseverative Errors

We conducted correlation analyses to further investigate variability between biological measures of stress and perseverative errors for the FAM S-R set with changed response contingencies. We found a negative correlation between the difference in heart rate from pre to during stress for females in the MP group (Spearman's ρ=-0.30, p=0.034); when examining the stress groups combined, this correlation was also statistically significant (Spearman's ρ=-0.45, p=0.009; Fig. 3.5A). This was not significant for females in the LP group (Spearman's ρ=0.24, p=0.22) or females within this cycle phase who were stressed (Spearman's ρ=0.13, p=0.42). Notably, the significant effect for females in the MP group is in the opposite direction to the effect in males; females in the MP group showed decreases in perseverative errors with increases in heart rate change. There were no significant correlations between peak cortisol level (20 min after stress) and perseverative errors in the MP (Spearman's ρ=0.11, p=0.44) or the LP group (Spearman's ρ=0.06, p=0.69); this was also not significant when considering females stressed in the MP (Spearman's ρ=0.02, p=0.93) or LP group (Spearman's ρ=0.24, p=0.22; Fig. 3.5B), and these results are comparable to males. We found no significant correlation between IBI variability

(lnRMSSD) and perseverative errors for females in the MP (Spearman's ρ=-0.07, *p*=0.61; Fig. 2.6) or LP groups (Spearman's ρ=-0.19, *p*=0.22); furthermore, there was no effect when considering the stress groups combined within the MP group (Spearman's ρ=0.13, *p*=0.49) or LP group (Spearman's ρ=-0.13, *p*=0.49; data not shown). We detected no significant correlations between the HF measure of HRV and perseverative errors for females in the MP group or the LP group (*p*'s>0.15; data not shown).

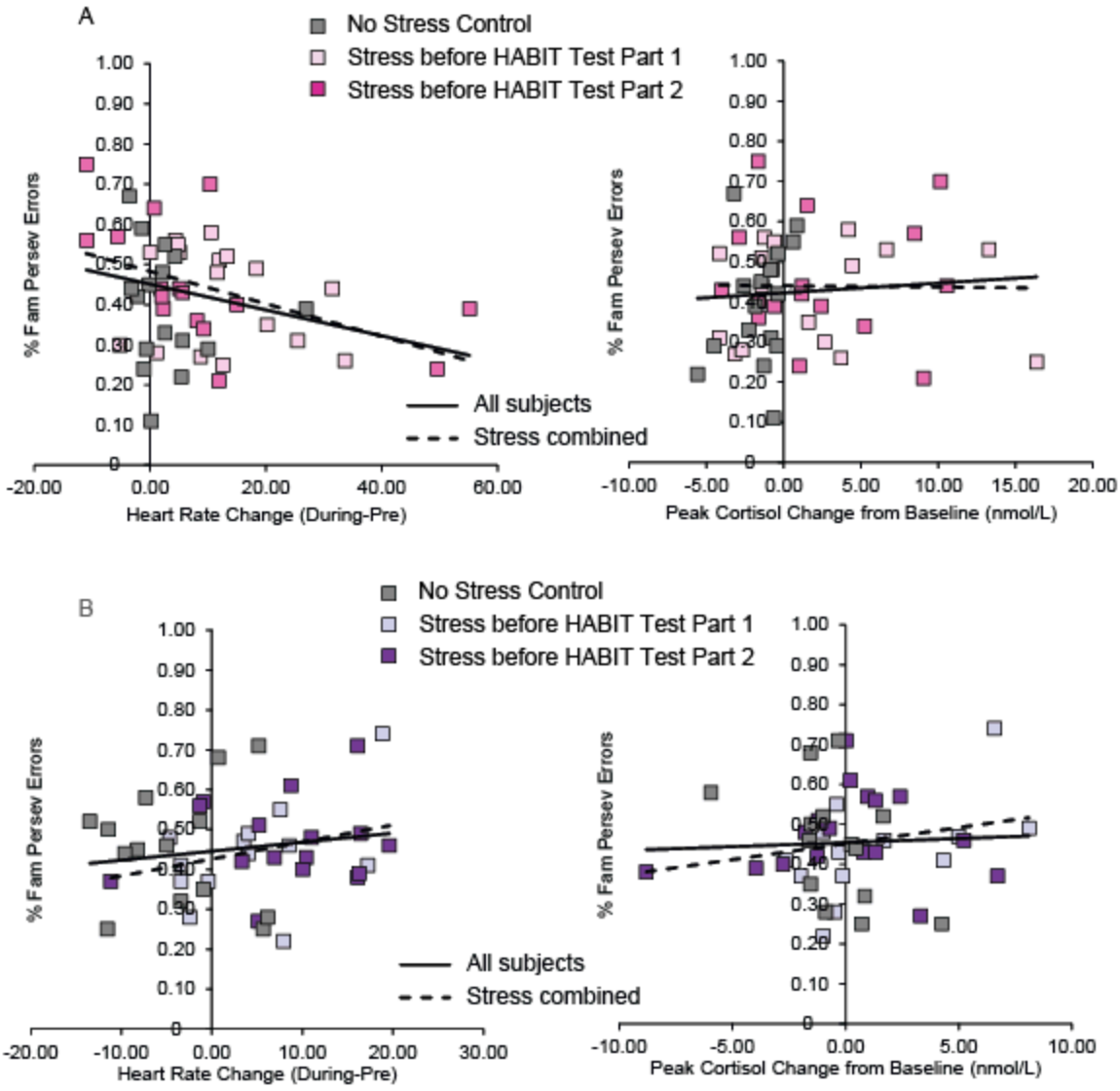

Figure 3.5. Correlations between biological measures of stress and perseverative errors. (A) Left: We found a negative correlation between the difference in heart rate (BPM) from pre to during stress for females in the MP group (Spearman's ρ=-0.30, *p*=0.034); when examining the stress groups combined, this correlation was also statistically significant (Spearman's ρ=-0.45, *p*=0.009). Right: Peak cortisol level (20 min after stress) and perseverative errors did not correlate in the MP group (Spearman's ρ=0.11, *p*=0.44) as a whole and it was also not significant when considering stressed females (Spearman's ρ=0.02, *p*=0.93). (B) Left: Heart rate change for females in the LP group was also not significantly correlated with perseverative errors (Spearman's ρ=0.24, *p*=0.22) or females within this cycle phase who were stressed (Spearman's ρ=0.13, *p*=0.42). (Spearman's ρ=0.06, *p*=0.69). Right: Peak cortisol did not correlate with perseverative errors for females in the LP group (Spearman's ρ=0.06, *p*=0.69) or when only considering females stressed within this group (Spearman's ρ=0.24, *p*=0.22)

Discussion

We examined the effect of menstrual cycle phase and stress timing of the SECPT on goal-directed and habitual responding in healthy females. In contrast to our earlier study with males (Chapter 2), we did not find differences in the timing of acute stress or interacting effects with menstrual cycle phase on our main index of habitual responding, perseverative errors. Additionally, we again found that perseverative behavior was independent of cortisol response. Our behavioral findings in this study do agree with some aspects of our study with males, for instance, stress did not impair S-R performance in either cycle phase. We did, however, find evidence that ovarian hormones may influence visuomotor performance. Specifically, females in the LP group showed higher accuracy for learning new S-R sets relative to females in the MP group; hormonal birth control use also, independently improved new S-R learning in the HABIT Part 1. We did also find hormonal contraceptive use to be associated with higher accuracy for NOV S-R sets after response devaluation. Together, these data suggest that higher ovarian hormone levels improve goal-directed response selection. Finally, we found that females in the stress before HABIT Test Part 1 re-learned new S-R contingencies more quickly, an effect that was independent of hormone status.

By varying the timing of our stress manipulation, we were able to test the effects of acute stress on both practiced S-R sets and the acquisition of newly introduced S-R associations. However, we did not find differences in habitual responding in our stress groups, and this was also not different between menstrual cycle phase groups. We did find evidence that SECPT exposure did not impair response inhibition, because females in the stress before HABIT Test Part 1 group were able to re-learn changed S-R contingencies more quickly. Interestingly, this provides further evidence to support our finding that stress did not shift behavior toward more habit-based

responding in females, in contrast to what we previously demonstrated in males (Chapter 2). Males that were stressed prior to HABIT Test Part 1 showed no impairments in performance until after response devaluation, at which point they perseverated significantly more frequently than those stressed before Part 2, or non-stressed controls. Furthermore, perseverative errors were positively correlated with change in heart rate in males and suggest that SECPT stress was detrimental to performance after response devaluation. In contrast, our female results suggest that increased changes in heart rate during stress (relative to baseline) are associated with decreases in perseverative errors for females in the MP group; there was no correlation for females in the LP group. Despite the opposite direction of the correlation in females relative to our male findings, these data provide further support that the effects of stress on task performance in our study are dependent upon activation of the SNS as opposed to HPA axis activation.

Additionally, we found enhancement effects of hormonal birth control on learning behavior pre-and post-devaluation for newly introduced S-R sets. The underlying mechanism of this enhancement is unknown, although this finding may reflect combinations of estrogen and progesterone derivatives on learning in general or on working memory. However, we did not find group differences in working memory assessed in a separate task (OSPAN). We did find that females using hormonal contraceptives showed a larger decrease in heart rate during stress. This marker of SNS activation suggests that the biological effects of the SECPT were more pronounced in these females. Although speculative, the combination of elevated ovarian hormones and stress may underlie our findings of enhanced task performance after response devaluation in females taking hormonal contraceptives. We did not directly assess ovarian hormone levels, but instead relied on self-report for verification of cycle phase. We were therefore not able to examine

variability in hormone levels that may correlate to behavior on various aspects of our HABIT task and cannot rule out effects of progesterone or estrogen, or both, on performance.

Our data demonstrated that elevations in both stress and ovarian hormones improve task performance. Research in rodents and nonhuman primates, as well as fMRI studies in humans, implicate the PFC as a target for estrogen modulation of cognitive function (Keenan et al., 2001; Rapp et al., 2003; Stevens et al., 2005; Joffe et al., 2006; Bohacek and Daniel, 2010; Toffoletto et al., 2014; Hara et al., 2016). Additionally, the PFC has also been shown to be important for enacting goal-directed behavior, and stress-induced shifts in behavior are correlated to decreased activity in this brain region (Schwabe et al., 2012). Our findings of enhancement in goal-directed response selection in females after stress, as well as increased performance by females using hormonal birth control, suggest that heightened levels of hormones may be acting within the PFC to modulate behavior. Moreover, we found that in the MP group, there was a negative correlation between changes in heart rate during stress (relative to baseline) and perseverative errors. This behavioral effect of stress points toward a beneficial effect of stress on overcoming well-trained responding; heightened levels of arousal may increase goal-directed behavior. Levels of ovarian hormones during the MP are lower and static relative to other cycle phases, suggesting the effects on performance may be driven by stress. In contrast, we did not find a correlation between perseverative errors and heart rate change for females in the LP group. Interestingly, the direction of that correlation was in the opposite direction in this group of females, albeit nonsignificant, and further suggests that synergistic effects of ovarian hormones and stress, such as higher ovarian hormone levels combined with stress, may result in impaired performance by increasing perseverative errors.

Our previous findings in males (Chapter 2) in addition to the results presented here from females, demonstrate that stress can be detrimental or beneficial to behavior. Furthermore, our female data suggest that ovarian hormones contribute to performance of goal-directed actions. Although the mechanism of action within the brain is undetermined, several lines of evidence support interactions between stress and ovarian hormones at the neural level. In humans, studies examining menopause or estrogen therapy on cognitive dysfunction have been inconclusive on the benefits of estrogen, most likely stemming from differences in outcome of interest, cognitive tasks assessed, and the role of other health-related variables such as stress (Shanmugan et al., 2016); studies in naturally cycling females have also found mixed effects of estrogen on cognitive function (Ossewaarde et al., 2010; Joseph et al., 2012; Thimm et al., 2014; Albert et al., 2015) or resting state brain function (Hjelmervik et al., 2014; Petersen et al., 2014), and of progesterone effects on functional connectivity between brains regions (Toffoletto et al., 2014; Arélin et al., 2015). The PFC has also been posited as the site of action for beneficial and detrimental stress effects on cognitive functions. In animal models, stress has been shown to have more detrimental effects on cognition in the presence of estrogen, demonstrating synergistic effects of stress and estrogen that can alter the threshold of stress necessary to result in PFC dysfunction (Shansky et al., 2004); however, rodent studies have also demonstrated that estrogen may be protective of chronic stress-induced deficits in PFC function (Kajantie and Phillips, 2006). These differences may reflect individual variability in response to stress (Yerkes and Dodson, 1908; Arnsten, 2009) and estrogen effects on cognitive function (Inagaki et al., 2010; Jacobs and D'Esposito, 2011) that have been posited to follow an inverted-U-shaped function in humans. This may manifest in individual variability in normal levels of catecholamines such as norepinephrine, serotonin, and dopamine; baseline levels of prefrontal dopamine assessed by catechol-O-methytransferase

(COMT) genotype, a putative marker of enzyme activity that has been shown to preferentially regulate dopamine breakdown in the PFC (Chen et al., 2004), interacts with estrogen to impact cognitive function in terms of working memory (Jacobs and D'Esposito, 2011) and impulsive choices (Smith et al., 2014). Baseline levels of stress and catecholamines may contribute to differences in cognitive function that are necessary for regulating goal-directed and habitual responses under stress.

We also demonstrate variability in subjective stress rating in 'unpleasantness' that was based on the timing of stress manipulation and did not vary with menstrual cycle phase. This subjective difference may be related to the interaction effect between menstrual cycle group and stress timing group on the duration of hand immersion during the SECPT. However, based on biological measures, the stress groups did not significantly differ in salivary cortisol or cardiac reactivity to stress that we used to examine changes in the biological sequelae over time. A possible explanation for the differences in the perception of the SECPT may relate to differences in how females versus males react to psychosocial stress. A recent study of women during cycle days 2-5 (matching the timing of our MP group) showed that free-cycling women in this phase were not different in stress response based on cardiac autonomic, negative mood, and anxiety responses to the TSST compared to women taking oral contraceptives (Villada et al., 2014). In contrast, during cycle days 2-5, women had larger decreases in positive mood after stress and higher anxiety overall. Thus, females in our study may have experienced these changes in emotion and mood, but we did not assess these factors via subjective questionnaire ratings of anxiety (e.g. STAI) before or after stress; we collected state and trait anxiety metrics (STAI) in the baseline Training session, which did not involve any stress manipulation, and the groups were not different prior to testing (Table 3.1). Furthermore, we did not assess behavioral coping strategies during or after stress via

self-report or behavioral observation, which could provide insight into gender differences in reaction to the stressor beyond biological analysis. These metrics could provide more fine-grained information regarding differences on individual variability to the stressor in women, or also determine whether women were more likely to displayed 'tend-and-befriend' behaviors as opposed to sympathetic activation that is related to fight or flight response that we previously demonstrated in males (Taylor et al., 2000).

We demonstrate differences in the actions of stress on goal-directed and habitual behaviors in males and females. Males were most likely to show perseverative responding after stress prior to HABIT Test Part 1, whereas females continued to engage in a goal-directed action strategy. Stress and ovarian hormones enhanced goal-directed action in females. These are the first studies to demonstrate the importance of stress timing in males, and specifically examine menstrual cycle effects on goal-directed and habitual behavior. Our behavioral findings demonstrate the necessity of future studies to examine direct levels of ovarian hormones on goal-directed and habitual behavior. Additional investigation of stress, and the potential for synergistic effects of stress and ovarian hormones, on the neural correlates of goal-directed and habitual action selection is warranted. Given the high prevalence of anxiety and mood disorders in women (Schiller et al., 2016), and exacerbation by stress, accounting for neuroendocrine effects of stress and ovarian hormones will have a broad impact on therapeutic benefit, as well as the opportunity for personalization of treatment.

CHAPTER 4

THE EFFECTS OF TRANSCRANIAL ALTERNATING CURRENT STIMULATION (TACS) ON HABITUAL RESPONDING IN ADDICTION

Introduction

The repetitive nature of the drug use cycle and disregard for action consequences are defining features of addiction. Ingrained behaviors become hard to change, and suggest that shifts in behavior could result from concomitant shifts in the dominance of neural control from top-down to bottom-up processes (Koob and Volkow, 2010). Evidence from animal and human studies suggest that this shift in top-down control can result from a decrease in PFC control over goal-directed behaviors, resulting in striatally driven response strategies that are bottom-up or habitual in nature (de Wit et al., 2012; Smith and Graybiel, 2013; McKim et al., 2016b). In animal models, exposure to alcohol or drugs of abuse can facilitate the transition to habitual responding (Corbit et al., 2012), with additional evidence suggesting that habitual responding after optogenetic inhibition of PFC leads to cocaine-seeking behavior despite footshock threat (Chen et al., 2013). In human studies, alcohol administration has also been shown to facilitate the use of a habit-based response strategy (Hogarth et al., 2012b), while studies of current or abstinent drug users are mixed. For example, several studies have shown goal-directed choice in smokers (Hogarth and Chase, 2011) or impairments in model-based (goal-directed) control of behavior in people diagnosed with alcohol dependence without changes in habitual or model-free action selection (Sebold et al., 2014); other studies have demonstrated either more habit-based responding in alcohol dependent people (Sjoerds et al., 2013), or no changes in model-based (goal) or model-

free (habitual) action selection in abstinent alcoholics compared to healthy controls (Voon et al., 2015). In amphetamine addiction, model-free action selection was the predominant choice strategy (Banca et al., 2016), and we and others have demonstrated that polysubstance addiction results in habit-based response selection (Ersche et al., 2016; McKim et al., 2016a). These behavioral findings from tasks directly assessing goal-directed and habitual behavior are supplemented by a single neuroimaging study in alcohol dependent participants that demonstrated deficits in PFC activation during action selection, which was associated with reduced goal-directed behavior, and enhanced habit-based responding (Sjoerds et al., 2013). Given extensive research and evidence from animal studies, in combination with mixed behavioral findings in humans, more specific testing of neural circuit control over action-selection behavior, particularly in people with SUDs, is warranted. Deficits in frontostriatal circuit control over behavior in addiction along with a propensity to form habits more rapidly may result in maladaptive behavior.

Research studies in humans have begun to probe the underlying neural basis of action selection in healthy control subjects using transcranial stimulation methods. For example, theta burst TMS to transiently inactivate the right DLPFC during the two-step decision task shifted choice behavior from a model-based to model-free strategy in healthy controls (Smittenaar et al., 2013), which is interpreted as evidence for more habitual action selection. Interestingly, this effect was dependent upon working memory capacity when TMS was applied over the left DLPFC; individuals with low working memory capacity showed the greatest reduction in model-based behavior, suggesting that high cognitive reserve (Stern, 2002) may protect against perturbations in brain function. Further work from this group tested the effects of anodal tDCs of the right DLPFC, predicted to enhance functioning of this brain region, and results demonstrated no effect of stimulation versus sham on model-based and model-free action selection (Smittenaar et al., 2014).

In addition to studies in healthy controls, a recent review of studies of TMS and tDCs brain stimulation methods for treatment and behavior change in addiction showed inconsistent results, potentially related to differences in substance studied, behavioral outcome of interest, or stimulation methodology (Salling and Martinez, 2016). These findings illustrate the need for research on novel methods to interrogate brain function that underlies goal-directed and habit behavior in general, as well as changes that may result from substance dependence.

An emerging non-invasive brain stimulation modality, tACs, applies weak oscillating currents to a brain region of interest to modulate endogenous cortical oscillations at the applied frequency. tACs has been used to determine causal links between cognitive function and behaviors (Thut et al., 2012; Herrmann et al., 2013), including domains of executive function, such as working memory (Meiron and Lavidor, 2014; Hoy et al., 2015), creative and abstract thinking (Lustenberger et al., 2015a), cognitive control during decision-making under risk (Sela et al., 2012), and reinforcement learning (Wischnewski et al., 2016). This method has further been tested in simulations of large-scale cortical networks to demonstrate that perturbations with tACs can switch brain networks between different activity states (Kutchko and Fröhlich, 2013); in the laboratory, application of tACs stimulation within the alpha range (~10 Hz) can enhance individual alpha EEG activity during and shortly after stimulation (Zaehle et al., 2010). Modulation of endogenous cortical rhythms to enhance the current cognitive 'state' could further be studied during behavioral task performance to measure the influence of these state changes on behavior, which may reduce imbalances at the network level that may be present in addiction (von Stein and Sarnthein, 2000).

Increasing evidence suggests that alpha rhythms (8-12 Hz) are important as a cognitive control mechanism to gate attention and focus on ongoing mental activity. It has been posited that

alpha band oscillatory activity reflects cortical idling and is most evident during a restful, waking state, and that alpha decreases during cognitive task engagement (Pfurtscheller et al., 1996). However, studies have begun to show increases in alpha activity during cognitive tasks (Buzsaki and Draguhn, 2004; Benedek et al., 2011; Lustenberger et al., 2015), further supporting that alpha activity in frontal brain regions is necessary for top-down inhibitory control, as proposed by the inhibition-timing hypothesis (von Stein and Sarnthein, 2000; Klimesch et al., 2007). Studies measuring EEG and behavioral correlates of executive function have demonstrated a relationship between frontal alpha activity and behavioral deficits in addiction. Frontal asymmetry in alpha frequency was related to increased choice of disadvantageous card decks in the Iowa Gambling Task (IGT) in cocaine addicts (Balconi and Finocchiaro, 2015), although changes in other frequency bands of the EEG measurements also suggest an overall imbalance that may not be specific to the alpha rhythm. Further studies have examined correlations between EEG measured at rest and task performance on the Tower of London Test (TLT) and Wisconsin Card Sorting Task (WCST). Results demonstrated that currently abstinent (medicated to minimize withdrawal) heroin abusers with the greatest alpha frequency power at rest performed worse on the TLT task, but no differences were found for perseverative errors on the WCST (Davydov and Polunina, 2004); these researchers also showed that fronto-central alpha frequencies were correlated with the duration of daily heroin abuse, but not abstinence, and this was also associated with decreased TLT performance (Polunina and Davydov, 2004). The evidence discussed above stemming from EEG and stimulation studies suggests that brain activity in the alpha band in frontal brain areas may represent an important target for improving or disrupting task performance requiring higher-level cognition.

Given the evidence of changes in alpha activity at rest in addiction, and recent evidence supporting the idea that alpha stimulation may increase cognitive flexibility (Lustenberger et al., 2015), we hypothesized that boosting alpha power via tACs may facilitate goal-directed action selection and reduce habitual actions. To test the hypothesis that enhancing top-down control shifts behavior toward a goal-directed, rather than habit-based, choice strategy, we examined the functional role of prefrontal alpha oscillations in coordinating and executing these behaviors. To do so, we tested otherwise healthy adults with an SUD history and a group of age, gender, and IQ matched control subjects in our HABIT paradigm in combination with tACs at alpha oscillation frequency to measure 10 Hz-tACs effects on goal-directed and habitual behavior response strategies, using a within-subject, active sham stimulation controlled, double-blind study design.

Methods

Participants

Healthy adults were recruited from the UNC Chapel Hill campus and surrounding community via advertisements. Participants (n=37) were aged 18-55 years old with no known history of neurological disorders, no current psychiatric diagnoses or psychoactive drug or medication use (excluding nicotine, alcohol and caffeine), and an estimated IQ within the normal range (≥80). Additional exclusion criteria included family history of epilepsy or seizures, current use of beta-blockers, brain implants/devices, colorblindness, history of brain surgery, or pregnancy. Participants were recruited into two groups based on whether they met DSM-V criteria for past drug or alcohol dependence in a structured clinical interview: healthy controls with no history of substance or alcohol dependence (Ctrl, n=20) or a history of alcohol or substance dependence but current abstinence (SUD, n=17). We additionally excluded participants in the SUD

group due to low IQ (*n*=3) and technical failure on the behavioral task (*n*=1). Participants in the SUD group were required to self-report ≥ 2 weeks of drug/alcohol abstinence prior to the initial study session; mean duration of abstinence was 1.5 yrs ± 2.5 yrs. Participants were screened for psychoactive drug use (Biotechnostix, Inc., Markham, ON), including alcohol (FC-10, Lifeloc Inc., Wheat Ridge, CO) at the start of each of the three study sessions. Each subject provided written informed consent as approved by the UNC Office of Human Research Ethics.

General Procedure

We used a randomized, double-blind within subjects design. Subjects participated in 3 sessions, with at least 1 night's sleep between each session. Subjects were paid for their participation, including performance bonuses in the Test sessions. During Session 0, participants underwent a structured clinical interview, completed a battery of standard questionnaires (see "Behavioral Inventories"), followed by behavioral training on the computerized S-R learning task (see "Behavioral Task"); no stimulation took place during Session 0 (Figure 2.1; Figure 4.1, HABIT Training). Participants also completed the automated OSPAN working memory task (Unsworth et al., 2005). Learning and habitual responding was then tested during Sessions 1 and 2 (stimulation sessions). During the stimulation sessions, participants completed the initial task practice and then Part 1 of the HABIT Test session (Figure 2.1). Head measurement and electrode placement was then performed, and participants completed Part 2 of the Test session while undergoing either tACs or active sham. During one of the two Test sessions, 10Hz-tACs was administered for the duration of the HABIT Test Part 2 (30 minutes); for the other session, 10Hz-tACs was administered for 5 minutes (active sham) at the beginning of the HABIT Test Part 2. The active sham condition was chosen to improve blinding to the neurosensory effects of the

stimulation parameters (Kanai et al., 2008; Turi et al., 2013; Raco et al., 2014). Each participant therefore received both active sham and true stimulation. All iterations of stimulation type were randomized and balanced between study groups (Ctrl and SUD). The number of days that elapsed between the HABIT Training and first (Ctrl: 3 ± 3 days; SUD: 5 ± 6 days; $t_{(35)}$=-1.18, p=0.25) and second Test session did not differ between groups (Ctrl: 6 ± 3.5 days; SUD: 8 ± 6 days; $t_{(35)}$=-1.45, p=0.16). The average time between stimulation sessions (Test sessions 1 and 2) was also not different between groups (Ctrl: 2.5 ± 2 days; SUD: 3 ± 2.5 days; $t_{(35)}$=-0.76, p=0.45). At the end of the session, participants completed a questionnaire regarding sensations and experience of the stimulation parameters for the session, and whether they believed they received stimulation (Fertonani et al., 2010). The experimental procedure is illustrated in Figure 4.1.

Behavioral Inventories

We administered a number of standard questionnaires to quantify factors that could impact our results. We quantified alcohol use behavior with the Alcohol Use and Disorders Identification test (AUDIT) (Saunders et al., 1993) and substance use behavior with the Drug Use Screening Inventory, Domain I (DUSI-I) (Tarter, 1990) and the Drug Abuse Screening Test (DAST) (Skinner, 1982). We calculated density of familial alcohol abuse using the Family Tree Questionnaire (FTQ) (Mann et al., 1985). Neuropsychological questionnaires included the Barratt Impulsivity Scale (BIS-11) (Barratt, 1994), the Beck Depression Inventory (BDI) (Beck and Steer, 1987), Rotter's Locus of Control scale (LOC) (Rotter, 1966), the State-Trait Anxiety Inventory (STAI) (Spielberger, 1985), the Thought Action Fusion scale (TAF) (Shafran et al., 1996) the Antisocial Practices (APS) of the Minnesota Multiphasic Personality Inventory 2 (MMPI-2) (Butcher JN et al., 1990) and the Perceived Stress Scale (Cohen et al., 1983). Education and

occupation were quantified with the Barratt Simplified Measure of Social Status (BSMSS; Barratt, 2006). We estimated IQ with the Shipley Institute of Living Scale (SILS; Zachary, 1991).

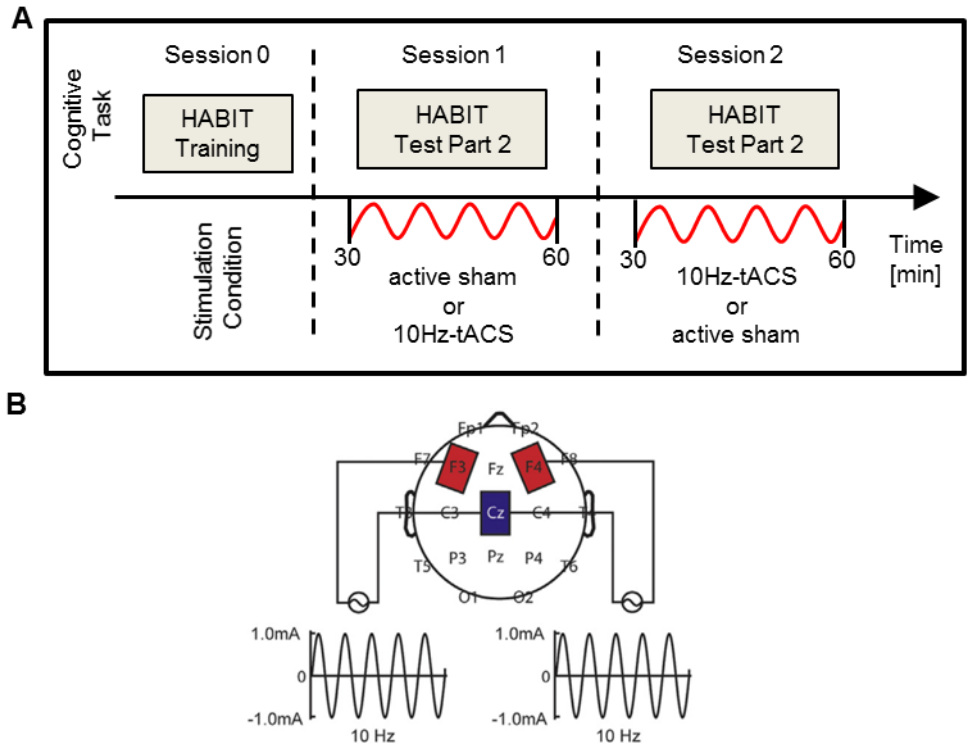

Figure 4.1. Study protocol and example electrode montage and stimulation parameters for bilateral DLPFC tACs. (A) Participants are randomized to receive either verum or active sham stimulation during HABIT Test Part 2 in a within subjects design. HABIT Test Part 2 task performance measures behavior after response devaluation, in which response contingencies change for a highly practiced and a newly learned S-R set. 10Hz-tACs was administered for the duration of the HABIT Test Part 2 (30 minutes); for the other session, 10Hz-tACs was administered for 5 minutes (active sham) at the beginning of the HABIT Test Part 2. (B) Placement of electrode locations via the 10-20 system of head measurement. The sham stimulation condition used a 5 min, 2mA peak-to-peak 10Hz sine-wave flanked by 10 second linear envelope ramps in and out for a total duration of 5 min and 20 seconds, following the methods of Lustenberger et al. (2015). True tACs stimulation used the same stimulation signal, but lasted 30 minutes instead of 5 minutes.

Behavioral Task

The HABIT is an S-R learning and re-learning task implemented in E-Prime 2.0 (PST Inc., Pittsburgh, PA) comprised of a HABIT Training session and a two part HABIT Test session, which

occurs on a subsequent day (Figure 2.1). Task details have been described previously (Boettiger and D'Esposito, 2005; McKim et al., 2016a). In brief, abstract visual stimuli were presented on a color LCD screen, and subjects used a four-button keypad for manual response selection using the fingers of their dominant hand. Participants were given instructions and a brief familiarization prior to completing the training phase of the task. Stimuli were displayed briefly (700 ms) on the screen, and participants learned through trial and error to associate stimuli with specific manual responses. During the Training session, participants learned two sets of S-R rules to a criterion of ≥ 90% accuracy (FAM sets). Participants then returned to the lab after ≥1 night's sleep to complete the Test session. In the Test session, participants first demonstrated retention of the previously learned (FAM) associations, then the learning task began (HABIT Test Part 1; Figure 2.1). In the learning task, blocks of the two FAM sets were interspersed with blocks composed of two new stimulus sets (NOV sets), to measure new S-R learning, and blocks of a control condition, consisting of novel, unrelated stimuli (No Rule set); blocks consisted of 15 randomly selected stimuli from the relevant set. Following 6 "runs" of 15 blocks each (3 per set-type), subjects were informed that the correct responses for two sets (one FAM and one NOV set) had changed (HABIT Test Part 2; Fig. 2.1). As the previously correct responses for the changed sets produce a negative rather than positive outcome, one could construe this change in response contingency as a response "devaluation," which is not to be confused with outcome devaluation procedures traditionally used in studies of habitual responding. This "response devaluation" manipulation allows us to quantify habitual responding when attempting to overcome both well-learned (FAM) and freshly learned (NOV) S-R associations, as the proportion of perseverative errors can be taken as an index of the degree to which responses are outcome independent (i.e. habit-based), as opposed to outcome-driven (i.e. goal-directed). By introducing S-R changes for both FAM and NOV sets, at a point

where performance is approximately equivalent, we can rule out performance deficits due to impaired response inhibition. Moreover, including FAM and NOV sets in which correct responses do not change allows us to control for effects on performance of time and of context change.

Transcranial alternating current stimulation (tACs)

Alternating current stimulation was delivered by a NeuroConn DC Stimulator Plus (NeuroConn, GmbH, Ilmenau, Germany) through three conductive rubber electrodes (CarboStim, Medstim Inc., Wabasha, MN). Participants underwent head measurement according to the international 10-20 system to place the three electrodes over the apex of the head (Cz) and the prefrontal cortex bilaterally (F3 and F4; Figure 4.1B). Two electrodes (4.45×4.45cm) were placed at F3 and F4, while the third, reference electrode was placed at Cz (4.45×9.53cm), and were securely adhered to the scalp with conductive paste (Ten20, D.O. Weaver, Aurora, CO, USA); impedance was kept below 5 kΩ. The sham stimulation condition used a 5 min, 2mA peak-to-peak 10Hz sine-wave flanked by 10 second linear envelope ramps in and out for a total duration of 5 min and 20 seconds, following the methods of Lustenberger et al. (2015). True tACs stimulation used the same stimulation signal, but lasted 30 minutes instead of 5 minutes. Frontal regions exhibit a peak in alpha activity during wakefulness around 10Hz (Tinguely et al., 2006), and frontal alpha band activity is thought to be involved in top-down control (Benedek et al., 2011; Klimesch et al., 2007). The participant was instructed that they would receive both real stimulation and sham stimulation, the order of which was randomized for the study sessions; the researcher and the participant were kept blind to the stimulation condition by pre-programming of the device settings by the PI.

Data Analysis

Our primary index of task performance in the HABIT Test sessions is accuracy. The HABIT is composed of six "runs" prior to response contingency change (Part 1), and an additional six runs after the contingency change (Part 2; Figure 2.1). We calculated accuracy in three time bins in Part 1, and three time bins in Part 2 by binning together two runs ("early", "mid", and "late") for each part. Where sphericity assumptions were violated for repeated measures analyses, we applied Greenhouse-Geisser corrections for degrees of freedom. In addition to considering accuracy, we also differentiated error types (perseverative responses, other incorrect responses) after the S-R contingency change to measure response-selection strategies utilized by participants. We also collected RT data in each trial. We tested for group differences in demographic and psychometric variables with unpaired two-tailed *t*-tests for continuous measures and χ^2 tests for categorical measures. All data analyses were performed in SPSS 22 (IBM Corp., Chicago, IL) or SAS 9.4 (SAS Institute Inc., Cary, NC).

Results

Demographic and Psychometric Data

Demographic questionnaire measures demonstrate that there were no significant differences between the SUD and Ctrl groups in terms of age, education, SES, estimated IQ, gender or ethnicity (Table 4.1). As expected, there were significant differences between groups in substance and alcohol use, with higher scores on all measures in the SUD group; the SUD group also reported a higher incidence of familial alcohol abuse (FTQ density; Table 4.1). Although the

results were not significant, the SUD group showed higher levels of perceived stress (a measure of cumulative life stress) and state anxiety (STAI-State) during the baseline Training session (Table 4.1). Thus, the SUD group tested here was somewhat older and reported somewhat lower Trait anxiety, but somewhat higher State Anxiety than the SUD group in our previous study (McKim et al., 2016a). Groups were also matched in terms of working memory measured via the automated OSPAN (all p's>0.25; Table 4.1), ruling out generalized deficits in executive function that could impact task performance.

Table 4.1. Sample Demographics and Psychometric Data Demonstrate Groups are Matched

	Ctrl Group	SUD Group	*t*(35)	*p*-value
Demographics	(*n*=20)	(*n*=17)		
Age (yrs)	38 ± 8	39 ± 9	-0.38	0.71
SILS (calculated) IQ	95 ± 6	93 ± 9	0.95	0.35
Education (yrs)	17 ± 2	15 ± 3	1.86	0.07
SES	52 ± 21	56 ± 16	-0.50	0.62
Gender (% female)	40	41	0.005	0.94§
Ethnicity (% non-white)	40	24	0.04	0.87#
Substance Use related				
AUDIT Total	2.5 ± 2	10 ± 11	-3.24	**0.003**
Consumption	2 ± 2	1.8 ± 4	-2.43	**0.033**
Dependence	0	0.39 ± 0.61	-2.25	**0.031**
Harm	0.20 ± 0.41	3.88 ± 4.27	-3.54	**0.003**
DUSI-I (%)	0.03 ± 0.06	0.74 ± 0.22	-12.99	**<0.001**
DAST	0.95 ± 0.69	18.06 ± 6.81	-10.32	**<0.001**
FTQ density (%)	0.06 ± 0.09	0.14 ± 0.11	-2.18	**0.04**
Psychometric				
Perceived Stress			-1.83	0.079
BIS Total			-1.78	0.086
Attention	12.89 ± 2.47	14.65 ± 4.33	-1.46	0.16
Motor	20.61 ± 3.15	22.82 ± 5.54	-1.44	0.16
Non-planning	20.50 ± 4.30	23.12 ± 5.13	-1.63	0.11
LOC	9.15 ± 3.28	9.59 ± 3.68	-0.38	0.70
STAI-State Anxiety	32.35 ± 5.05	37.00 ± 9.39	-1.92	0.064
STAI-Trait Anxiety	28.75 ± 6.39	33.00 ± 8.95	-1.68	0.10
TAF Total	19.85 ± 13.65	17.00 ± 12.55	0.66	0.52
Moral	17.60 ± 11.87	15.94 ± 12.23	0.42	0.68
Self	1.65 ± 2.68	0.82 ± 1.51	1.18	0.25
Others	0.60 ± 1.76	0.24 ± 0.75	0.79	0.43
MMPI-Antisocial	6.10 ± 4.35	7.65 ± 3.16	0.78	0.46
Working Memory				
OSPAN Score	38.10 ± 16.89	38.06 ± 16.84	0.007	0.99
OSPAN Total	56.05 ± 12.65	53.82 ± 14.32	0.50	0.62
Accuracy Errors	7.20 ± 5.18	5.47 ± 3.54	1.16	0.25
Math Errors	8.70 ± 7.34	7.06 ± 3.63	0.84	0.41
Speed Errors	1.50 ± 2.54	1.53 ± 1.42	-0.04	0.97

Values are reported as mean ± standard deviation. Reported *p*-values reflect the results of unpaired two-tailed comparison between groups. IQ, Intelligence Quotient; SES, Socioeconomic Status; AUDIT, Alcohol Use Disorders Identification Test; DUSI-I, Drug Use Screening Inventory, Domain I; DAST, Drug Abuse Screening Test; FTQ, Family Tree Questionnaire; Barratt Impulsivity Scale; LOC, Locus of Control; SILS, Shipley Institute of Living Scale; STAI, State-Trait Anxiety Inventory; TAF, Thought Action Fusion Scale. MMPI-Antisocial, Minnesota Multiphasic Personality Inventory. §*p*-value represents result of Chi-square test. #*p*-value represents result of Fischer's exact test. **Boldface** indicates significant difference between groups.

Behavioral Performance during HABIT Training

During the HABIT Training session, participants were required to reach a performance criterion of 90% accuracy for each (FAM) set. Training to criterion took ~25 min, with no significant differences between groups in the average number of training blocks needed to learn the first ($t_{(35)}$=-1.95, p=0.07) or second ($t_{(35)}$=-0.76, p=0.45) FAM set. Participants next completed a third practice version of the task that switched between blocks of FAM sets 1 and 2 and were required to reach 70% accuracy; blocks to criterion did not differ by group ($t_{(35)}$=0.61, p=0.55). Thus, training performance between groups was equivalent prior to returning for the HABIT Test session. Participants demonstrated retention of previously learned FAM sets by again reaching the performance criterion of 70% at the start of each Test session by repeating the practice version that includes blocks of both FAM sets. Groups did not differ in the number of trials to reach this criterion the first ($t_{(35)}$=-0.17, p=0.87) or second Test session ($t_{(35)}$=-1.00, p=0.33).

Stimulation Blinding and Subjective Effects of Stimulation

Chi-square analysis confirmed that the randomization order of the stimulation conditions was counterbalanced across groups ($\chi^2_{(1)}$ = 0.70, p=0.40). Participants were successfully blinded to the stimulation conditions, and blinding success did not differ between groups for either true 10Hz-tACS (Ctrl group: 12/20 correct; SUD group: 10/17 correct: $\chi^2_{(1)}$ = 0.005, p=0.94) or sham stimulation (Ctrl group: 12/20 correct; SUD group 5/17 correct; $\chi^2_{(1)}$ = 3.46, p=0.063). Subjective report of sensations for active versus sham stimulation also did not differ between groups (all *p's*>0.13) (Fig. 4.2).

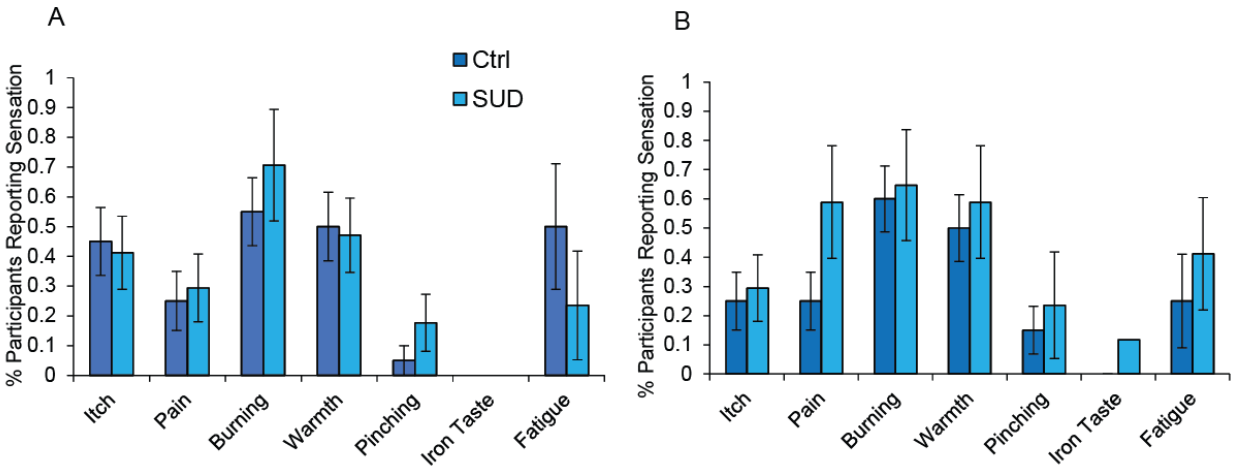

Figure 4.2 Percentage of participants reporting sensations during active stimulation and sham stimulation. Results demonstrated no significant differences between groups for (A) true versus (B) sham stimulation, showing similar subjective experience of both conditions.

Test Session Part 1: Learning New S-R Sets and Execution of Familiar S-R Sets

To assess performance during Part 1 of the HABIT Test sessions, we conducted a mixed model repeated measures ANCOVA with set-type (FAM/NOV), time (early, mid, late), and stimulation condition (true, sham) as within subject factors, group (SUD/Ctrl) as a between subject factor, and stimulation order as a covariate. Although no stimulation (true or sham) took place during Part 1 of the Test sessions, we include stimulation condition as a factor to verify that Part 1 performance was well matched for each session. We found expected main effects of set-type, with higher accuracy for FAM versus NOV sets ($F_{(1,34)}$=11.66, p=0.002, η^2=0.06; Fig. 4.3), and time, with accuracy improving from early (0.70 ± 0.02) to mid (0.77 ± 0.02) to late (0.80 ± 0.02) runs ($F_{(1.44,49.98)}$=9.19, p<0.001, η^2=0.01; Fig. 4.3). We also found a significant set-type×time interaction ($F_{(1.43,48.77)}$=9.07, p=0.001, η^2=0.01), reflecting a greater improvement of NOV set performance over time (Fig. 4.3). We found a trend toward a significant interaction between time and group ($F_{(1.44,48.98)}$=3.07, p=0.071, η^2=0.004), with larger increases in accuracy over time for the SUD group relative to the Ctrl group. There was a main effect of stimulation condition

($F_{(1,34)}$=22.40, p<0.001 η^2=0.10), demonstrating that accuracy was higher when participants were randomized to receive active stimulation relative to sham stimulation. Stimulation condition also interacted with stimulation order ($F_{(1,34)}$=23.05, p<0.001 η^2=0.11), suggesting higher accuracy when active stimulation occurred during the second test session relative to the first; during sham stimulation, accuracy was higher when sham occurred during the second test session relative to the first test session.

To decompose the significant interaction between set-type and time, we ran separate repeated measures ANCOVAs to evaluate time and group, controlling for stimulation order, for each set-type. Performance was stable over time for the FAM sets and did not improve overall ($F_{(1.59,52.61)}$=1.24, p=0.29); there was a trend for an interaction between time and group ($F_{(1.59,52.61)}$=3.16, p=0.061). The Ctrl group tended to show a larger change in accuracy from early to mid that stabilized over time, whereas the SUD group steadily increased in accuracy over time. There was also a stimulation condition by stimulation order interaction ($F_{(1,33)}$=35.56, p<0.001), again demonstrating that accuracy was higher for active stimulation during test session 2, relative to receiving active stimulation during test session 1; for sham stimulation, accuracy was slightly higher during test session 1 relative to receiving sham stimulation during test session 2. Stimulation order did not interact with time or group (all F's<1.31, all p's>0.28). Consistent with our published data and the studies discussed in the previous chapters, accuracy increased significantly with time for the NOV sets ($F_{(1.40,47.45)}$=12.40, p<0.001; Fig. 4.3). We detected no main effect of group on NOV set learning ($F_{(1,33)}$=1.19, p=0.28) nor an interaction between time and group ($F_{(1.40,47.45)}$=2.10 p=0.15). There was a main effect of stimulation condition on NOV learning ($F_{(1,34)}$=6.38 ,p=0.016), demonstrating increased accuracy overall for NOV sets during the active stimulation test sessions. There was also a significant stimulation condition by stimulation order interaction ($F_{(1,34)}$=5.77,

p=0.022). Again, accuracy was higher for active stimulation during test session 2, relative to receiving active stimulation during test session 1; for sham stimulation, accuracy was higher during test session 1 relative to receiving sham stimulation during test session 2. Together, these data replicate our previous results and further demonstrate that the SUD group is not impaired in execution or learning of S-R sets relative to controls.

We further assessed changes in learning in terms of RTs, conducting an identical ANCOVA to that described above, but taking RT as the dependent measure. We did not find a significant main effect of set-type ($F_{(1,34)}$=1.30, p=0.26), demonstrating similar RTs for both sets. There was also no significant effect of time ($F_{(1.44,48.86)}$=0.20, p=0.82; data not shown), further suggesting stable RTs over time. There was a trend for a set-type by time interaction ($F_{(1.55,52.56)}$=2.82, p=0.081, η^2=0.006), such that RTs for FAM sets decreased at a faster rate over time relative to NOV sets. There was a main effect of stimulation condition ($F_{(1,34)}$=6.63, p=0.015, η^2=0.05), demonstrating that accuracy was higher when participants received active stimulation relative to sham. A significant stimulation condition by set-type by time interaction ($F_{(1.00,1.88,63.91)}$=6.72, p=0.003, η^2=0.008), demonstrated that during active stimulation, RTs decreased faster over time for NOV sets relative to FAM sets. In contrast, during sham stimulation, RTs decreased faster over time for FAM sets relative to NOV sets. Stimulation condition also interacted with stimulation order ($F_{(1,34)}$=6.64, p=0.014, η^2=0.05), suggesting faster RTs when active stimulation occurred during the second test session relative to the first; during the sham stimulation sessions, RTs were faster when sham occurred during the second test session relative to the first test session. Group interacted with stimulation condition, set-type, and time ($F_{(1.00,1.88,63.91)}$=5.45, p=0.008, η^2=0.007), demonstrating that the Ctrl group showed lower and decreasing RTs over time for FAM sets regardless of stimulation condition, whereas NOV set RTs

significantly decreased over time during the active stimulation session relative to sham stimulation. For the SUD group, larger decreases in RTs over time were evident for NOV sets during the active stimulation session; FAM set RTs decreased at a steeper rate over time during the sham session relative to the active stimulation session. We also found a significant stimulation condition×set-type×time×stimulation order interaction ($F_{(1.00,1.00,1.88,63.91)}$=3.48, p=0.04, η^2=0.004).

To decompose the significant higher order interaction, we ran separate repeated measures ANCOVAs to evaluate time and group, controlling for stimulation order, for each set-type. Performance was stable over time for the FAM sets and did not improve overall ($F_{(1.42,48.18)}$=0.72, p=0.49). There was a main effect of stimulation condition ($F_{(1,34)}$=16.50, p<0.001), showing that RTs were slightly faster overall for FAM sets during the sham stimulation session. There was also a stimulation condition by stimulation order interaction ($F_{(1,34)}$=18.74, p<0.001), demonstrating that RTs were faster for the active stimulation condition during test session 2, relative to receiving active stimulation during test session 1; for sham stimulation, RTs were faster during test session 2 relative to receiving the sham stimulation condition during test session 1. A significant interaction between stimulation condition, time and group ($F_{(1.00,1.92,65.31)}$=3.55 p=0.036) showed that the Ctrl group showed larger decreases in RTs over time during the active stimulation session relative to the sham stimulation session; in the SUD group, RTs decreased faster over time during the sham stimulation session. For NOV sets, there were no significant effects of time, stimulation condition, or group (all F's<2.18, p<0.12). There was a significant interaction between stimulation order and time ($F_{(1.50,50.98)}$=3.53 p=0.045), suggesting that RTs decreased at a faster rate when active stimulation occurred during the first test session, relative to stable RTs over time when active stimulation occurred during the second test session. Overall, RT results further confirm our

accuracy findings by demonstrating that the SUD group is not impaired at executing and performing S-R sets over time.

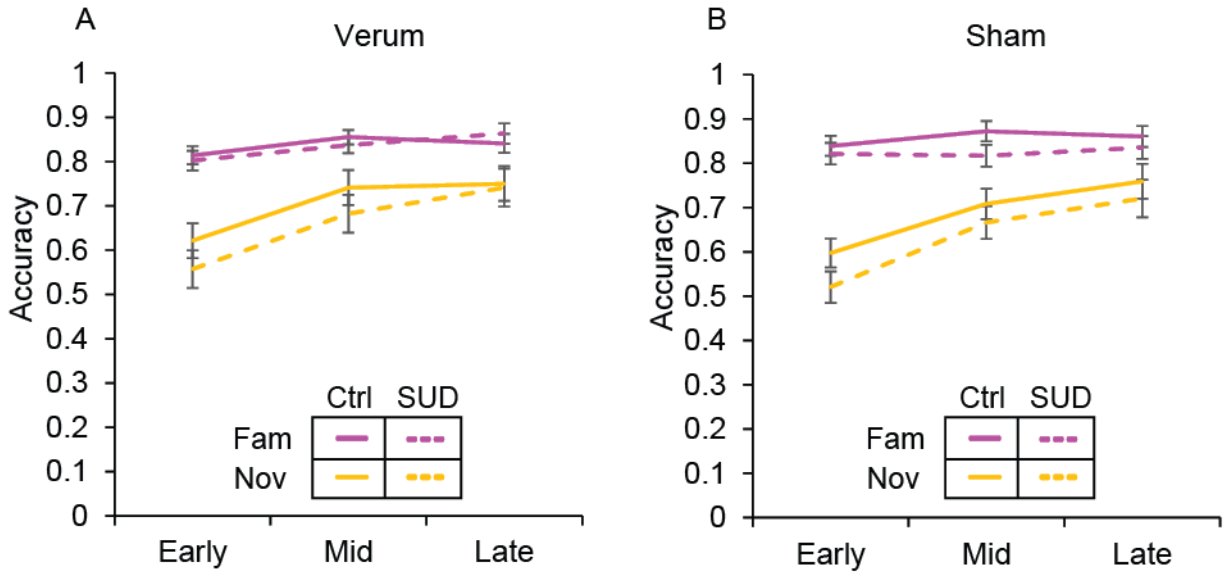

Figure 4.3. Performance for FAM and NOV sets by group during HABIT Test Part 1 based on within subject randomization to stimulation condition (verum (A) vs. sham (B)). Note, no stimulation (true or sham) took place during Part 1 of the Test sessions, but we included stimulation condition as a factor to verify that Part 1 performance was well matched for each session. We found a significant set-type×time interaction ($F_{(1.43,48.77)}$=9.07, p=0.001, η^2=0.01), reflecting a greater improvement of NOV set (yellow lines) performance over time. We found a trend toward a significant interaction between time and group ($F_{(1.44,48.98)}$=3.07, p=0.071, η^2=0.004), with larger increases in accuracy over time for the SUD group (dashed lines) relative to the Ctrl group (solid lines). Performance was stable over time for the FAM sets and did not improve overall ($F_{(1.59,52.61)}$=1.24, p=0.29; purple lines in A & B); there was a trend for an interaction between time and group ($F_{(1.59,52.61)}$=3.16, p=0.061). The Ctrl group (solid lines) tended to show a larger change in accuracy from early to mid that stabilized over time, whereas the SUD group (dashed lines) steadily increased in accuracy over time. Accuracy increased significantly with time for the NOV sets ($F_{(1.40,47.45)}$=12.40, p<0.001; yellow lines). We detected no main effect of group on NOV set learning ($F_{(1,33)}$=1.19, p=0.28) nor interaction between time and group ($F_{(1.40,47.45)}$=2.10 p=0.15).

Test Session Part 2: Behavioral Performance on Familiar and Novel S-R Sets

To evaluate task performance post-contingency change, we first conducted a mixed model ANCOVA with within subject factors of stimulation type (verum or sham), set-type (FAM or NOV set), contingency change (yes or no), and time (early, mid, late), with group as the between subject factor, and stimulation order as a covariate. We detected a significant main effect time ($F_{(1.61,54.85)}$=8.42, p=0.001, η^2=0.01; Figure 4.4), suggesting that accuracy improved over time after response devaluation. We did not find a significant main effect of set-type ($F_{(1,34)}$=0.18, p=0.67), suggesting that overall accuracy by the end of the six runs was equivalent between FAM and NOV sets. A main effect of stimulation condition ($F_{(1,34)}$=7.89, p=0.008, η^2=0.02) revealed that active stimulation resulted in higher accuracy on the task overall. We found a main effect of contingency change ($F_{(1,34)}$=7.73, p=0.009, η^2=0.02), demonstrating lower accuracy overall for sets with changed response contingencies relative to sets that did not change. Contingency change also interacted with time ($F_{(1.72,58.60)}$=9.46, p=0.001, η^2=0.008), demonstrating larger changes in accuracy over time for sets that changed relative to more stable performance over time on sets that did not change. We observed a trend for a contingency change by set-type interaction ($F_{(1,34)}$=3.96, p=0.055, η^2=0.008; Fig. 4.4), suggesting that in the FAM sets, accuracy for the set with changed response contingencies was slightly higher relative to the unchanged contingency set, whereas in the NOV set, the set with changed response contingencies had lower accuracy overall compared to the set that did not change. We did not find any significant main effects or interactions with group (all F's<2.61, all p's>0.12). We also found a higher order interaction between set-type, contingency change, time and stimulation order ($F_{(1.00,1.72,1.86,63.09)}$=3.53, p=0.038, η^2=0.002), which we further interrogate below.

To decompose this interaction, we ran separate repeated measures ANCOVAs for each set-type including the same within and between factors as above. Performance improved over time for the FAM sets ($F_{(2,68)}$=7.34, p=0.001, η^2=0.02), particularly from the early to mid time points ($F_{(1,34)}$=9.05, p=0.005). We also found a main effect of contingency change ($F_{(1,34)}$=16.24, p<0.001, η^2=0.09), demonstrating that accuracy was only slightly higher overall for sets with changed response contingencies relative to sets that did not change responses. We found an interaction between contingency change and time ($F_{(2,68)}$=10.61, p<0.001, η^2=0.02; Fig. 4.4), showing larger increases in performance over time for sets with changed contingencies after response devaluation. There was a main effect of stimulation condition ($F_{(1,34)}$=12.03, p=0.001, η^2=0.05), with higher accuracy for verum stimulation versus sham. Stimulation condition also interacted with contingency change ($F_{(1,34)}$=4.54 p=0.041, η^2=0.01), demonstrating higher accuracy for unchanged sets during active stimulation versus higher accuracy for changed sets during sham stimulation. We detected a trend for a stimulation by group interaction ($F_{(1,34)}$=3.49, p=0.070, η^2=0.01), such that performance was elevated during sham stimulation in the Ctrl group, while performance was higher in the SUD group during the verum condition. A trend was also evident for a three-way interaction between stimulation condition, contingency change, and group ($F_{(1,1,34)}$=3.58, p=0.067, η^2=0.01). This reflected that performance on sets with changed response contingencies was higher under sham stimulation for controls, whereas sets that did not change contingencies showed higher accuracy during the verum condition. The SUD group showed similar increased performance overall on sets with changed contingencies during sham stimulation, but the magnitude of this difference was more pronounced; they also performed better overall on sets without contingency change during active stimulation. Stimulation condition and stimulation order also interacted ($F_{(1,34)}$=9.60 p=0.001, η^2=0.04), such that accuracy was higher

overall when active stimulation occurred in the first test session relative to the second, whereas accuracy was lower in the sham condition if it occurred during the first study session relative to when it occurred the second. We also found interactions between contingency change and stimulation order ($F_{(1,34)}$=19.55, p<0.001, η^2=0.11) that demonstrated higher accuracy levels for unchanged contingency sets when sham stimulation occurred first, but higher levels of accuracy for changed contingency sets if active stimulation occurred first. Furthermore, stimulation order also interacted with contingency change and time ($F_{(1,2,68)}$=8.27, p=0.001, η^2=0.02), showing that during the second testing session, performance was most similar between sets with changed contingencies under active stimulation and sets with unchanged contingencies during sham stimulation; in contrast, during the first stimulation session, accuracy performance was most similar for unchanged sets under sham stimulation and sets with changed contingencies over time during active stimulation. Performance increased over time for NOV sets after response devaluation ($F_{(1.56,53.08)}$=5.03, p=0.009, η^2=0.01), reflecting larger changes from the mid to late time points ($F_{(1,34)}$=5.84, p=0.021), but not early to mid time points ($F_{(1,34)}$=1.92, p=0.174). There were no effects or interactions with stimulation condition (all F's<1.98, all p's>0.18) or group (all F's<2.01, all p's>0.17).

We also evaluated task performance post-contingency change based on RTs to determine whether our stimulation conditions changed this metric of performance. We conducted an identical ANCOVA to that described above, but taking RT as the dependent measure. A main effect of set-type ($F_{(1,34)}$=5.18, p=0.029, η^2=0.01) revealed that RT was slower for NOV sets relative to FAM sets. We found an interaction between set-type and contingency change ($F_{(1,34)}$=9.92, p=0.003, η^2=0.008), demonstrating that RTs were faster overall for unchanged sets, but for both changed and unchanged sets, FAM set RTs were faster compared to NOV set RTs. We also found a trend for

a set-type by contingency change by time interaction ($F_{(1,2,68)}$=2.76, p=0.071 η^2=0.002) suggesting a stable decrease in RT over time for unchanged sets, whereas the largest decreases in RTs for changed sets occured from the beginning to middle of task performance. There was a trend for a contingency change by group interaction ($F_{(1,34)}$=3.44, p=0.072, η^2=0.004), such that there was greater slowing for the changed sets relative to the unchanged sets in the Ctrl group compared to the SUD group. We also found a higher order interaction between stimulation condition, set-type, contingency change, time, and stimulation order ($F_{(1,1,1,34)}$=5.68, p=0.006, η^2=0.005; data not shown).

To further probe this interaction, we ran separate repeated measures ANCOVAs for each set-type including the same within and between factors specified above. For FAM set RTs, there was a significant main effect of contingency change ($F_{(1,34)}$=8.91, p=0.005, η^2=0.03) that demonstrated slower RTs for sets with changed response contingencies relative to unchanged sets. Stimulation condition interacted with contingency change ($F_{(1,34)}$=6.52, p=0.015, η^2=0.09), which showed increased RTs for changed response sets relative to sets without changed responses, regardless of stimulation condition; the RT slowing effect was more pronounced in the active stimulation condition. An interaction between stimulation condition, contingency change and stimulation order ($F_{(1,1,34)}$=6.82, p=0.0153, η^2=0.09) showed that when active stimulation occurred during the second test session, RTs were faster for unchanged contingency sets relative to changed contingency sets. In contrast, active stimulation in the first test session resulted in slightly slowed RTs for the unchanged contingency sets. When sham stimulation occurred during the first test session, RTs were slowed for sets with changed contingencies relative to unchanged sets, and this difference was even more pronounced when sham stimulation occurred during the second test session. There were no main or interacting effects of group for FAM set RTs (all F's<1.44,

p's>0.24). NOV set RTs demonstrated a significant simulation condition by contingency change by time interaction ($F_{(1,1,34)}$=4.88, p=0.010, η^2=0.02). This effect was driven by larger decreases in RT over time for changed contingency sets from the beginning to middle task performance, while there was a steady decline in RTs over time for the unchanged contingency sets during sham stimulation. During active stimulation, there were larger changes over time in RTs for changed contingency sets, relative to a steady decrease in RTs for unchanged response sets. There were no main effects or interactions with group (all F's<0.39, all p's>0.69).

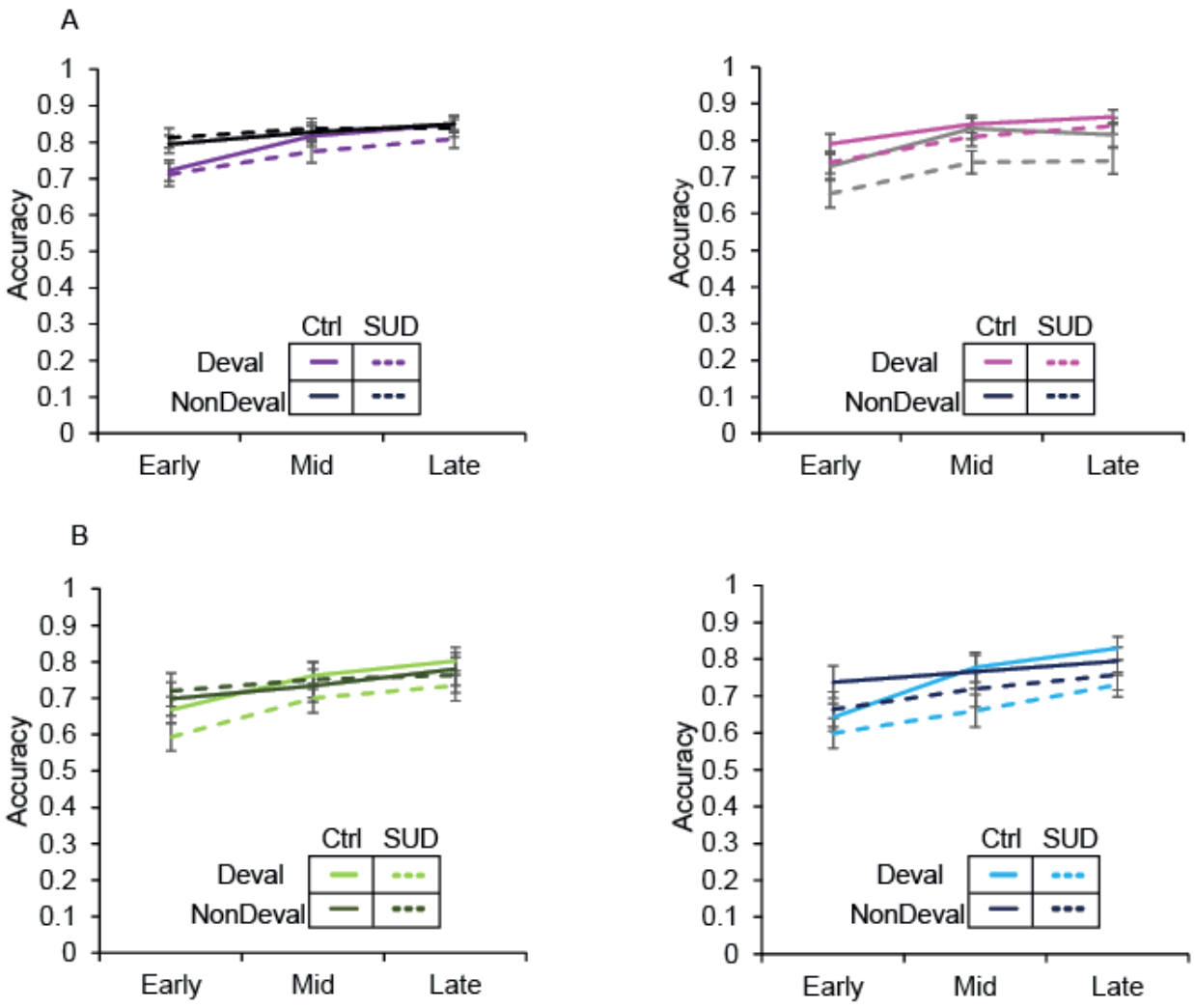

Figure 4.4. Performance for FAM and NOV sets after response devaluation during HABIT Test Part 2. Left column images depict performance during true stimulation and right column depicts performance during active sham. Top panel (A) depicts FAM sets and bottom panel (B) depicts NOV sets. Overall, accuracy improved over time after response devaluation ($F_{(1.61,54.85)}$=8.42, p=0.001, η^2=0.01). We observed a trend for a contingency change by set-type interaction ($F_{(1,34)}$=3.96, p=0.055, η^2=0.008), suggesting that in the FAM sets, accuracy for the set with changed response contingencies ("Deval" purple (left) and pink (right)) was slightly higher relative to the unchanged contingency set ("NonDeval", black (left) and grey (right)). In the NOV set, the set with changed response contingencies ("Deval" light green (left) and light blue (right)) had lower accuracy overall compared to the set that did not change ("NonDeval" dark green (left) and dark blue (right)). (A) Performance improved over time for the FAM sets ($F_{(2,68)}$=7.34, p=0.001, η^2=0.02). We found an interaction between contingency change and time ($F_{(2,68)}$=10.61, p<0.001, η^2=0.02), showing larger increases in performance over time for sets with changed contingencies after response devaluation. There was a main effect of stimulation condition ($F_{(1,34)}$=12.03, p=0.001, η^2=0.05; left versus right column), with higher accuracy for verum stimulation (A, left side) versus sham (A, right side). Stimulation condition also interacted with contingency change ($F_{(1,34)}$=4.54 p=0.041, η^2=0.01), demonstrating higher accuracy for unchanged sets during active stimulation versus higher accuracy for changed sets during sham stimulation. We detected a trend

for a stimulation by group interaction ($F_{(1,34)}$=3.49, p=0.070, η^2=0.01), such that performance was elevated during sham stimulation in the Ctrl group (solid lines), while performance was higher in the SUD group (dashed lines) during the verum condition. A trend was also evident for a three-way interaction between stimulation condition, contingency change, and group ($F_{(1,1,34)}$=3.58, p=0.067, η^2=0.01). This reflected that performance on sets with changed response contingencies was higher under sham stimulation for controls, whereas sets that did not change contingencies showed higher accuracy during the verum condition. The SUD group showed similar increased performance overall on sets with changed contingencies during sham stimulation, but the magnitude of this difference was more pronounced; they also performed better overall on sets without contingency change during active stimulation. (B) Performance increased over time for NOV sets (bottom) after response devaluation ($F_{(1.56,53.08)}$=5.03, p=0.009, η^2=0.01), reflecting larger changes from the mid to late time points ($F_{(1,34)}$=5.84, p=0.021), but not early to mid time points ($F_{(1,34)}$=1.92, p=0.174). There were no effects or interactions with stimulation condition (all F's<1.98, all p's>0.18) or group (all F's<2.01, all p's>0.17).

Habitual Responding: Quantifying Perseverative Errors Post-Contingency Change

To quantify the degree to which responses were habitual, we calculated the percentage of perseverative errors relative to total errors following S-R contingency change (McKim et al., 2016). A stimulation condition by group mixed model ANCOVA, covarying for stimulation order, found a significant interaction between stimulation condition and group on perseverative errors for the FAM set with changed response contingencies ($F_{(1,33)}$=4.33, p=0.045, η^2=0.10). To decompose this interaction, we used the Wilcoxon signed ranks test to assess the difference between perseverative errors for each stimulation type within group. Results demonstrated more perseverative errors committed by the Ctrl group under true 10Hz-tACS versus sham stimulation (z=-1.97, p=0.049). In contrast, there was no significant difference in perseverative errors for the SUD group under true stimulation or active sham (z=-0.75, p=0.45; Fig. 4.5). We also found a significant interaction between stimulation condition and order ($F_{(1,33)}$=6.28, p=0.045, η^2=0.14), which suggests that participants that received active stimulation during Test session 1 had higher perseverative errors than those who received active stimulation in Test session 2. Individual

differences in active versus sham stimulation on FAM perseverative responding is illustrated in Figure 4.6.

We next tested whether perseverative errors were stable over time or if they could be overcome during the post-contingency re-learning period (i.e. HABIT Test Part 2). A stimulation condition by group mixed model ANOVA including time, covarying for stimulation order, showed a significant main effect of time ($F_{(1,66)}$=4.64, p=0.013, η^2=0.05), such that perseverative errors were likely to decrease over time; this did not interact with group ($F_{(2,66)}$=1.21, p=0.30, η^2=0.01), suggesting that the rate of decrease over time was similar between Ctrl and SUD groups (Figure 4.5B). There was a trend for a significant interaction between stimulation condition and group ($F_{(1,33)}$=3.58, p=0.067, η^2=0.03), demonstrating increased perseverative errors in the Ctrl group relative to the SUD group during true stimulation, but the opposite pattern during sham stimulation. There were no main or interacting effects of stimulation order (all F's<2.26, all p's>0.14).

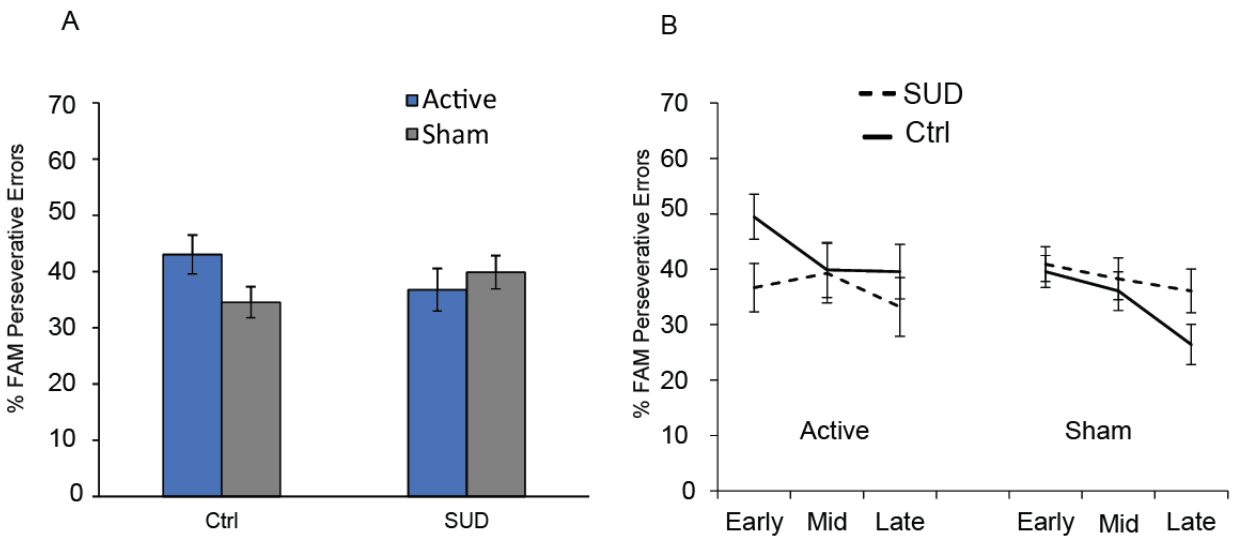

Figure 4.5. Total percentage of perseverative errors and change over time by stimulation condition. (A) Results demonstrated a significant stimulation condition by group interaction for the FAM set with changed response contingencies ($F_{(1,33)}$=4.33, p=0.045, η^2=0.10). The Ctrl group perseverated more under active stimulation, while perseverative errors were not significantly different between stimulation conditions for the SUD group. (B) A significant main effect of time ($F_{(1,66)}$=4.64, p=0.013, η^2=0.05) showed that perseverative errors were likely to decrease over time; this did not interact with group ($F_{(2,66)}$=1.21, p=0.30, η^2=0.01). There was a trend for a significant interaction between stimulation condition and group ($F_{(1,33)}$=3.58, p=0.067, η^2=0.03), suggesting that true stimulation reduced perseverative errors for the SUD group, but increased perseverative errors in the Ctrl group relative to sham stimulation

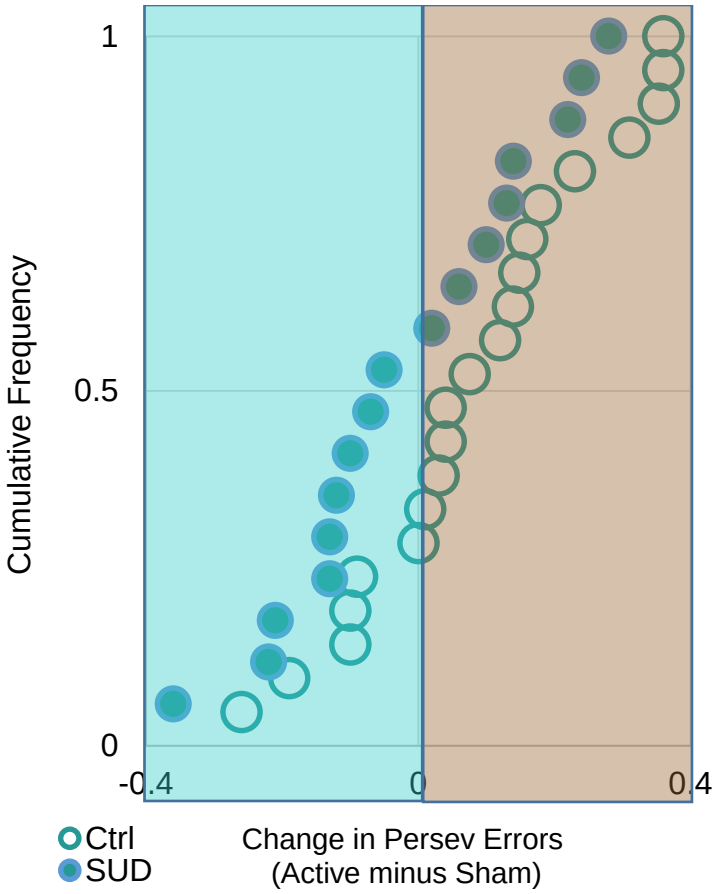

Figure 4.6. Cumulative distribution frequency plot for FAM set change in perseverative errors by group. Filled circles depict SUD individuals; open circles depict Ctrl group individuals. Y-axis indicates the cumulative frequency. X-axis indicates the change in perseverative errors in the verum stimulation condition minus the sham stimulation condition. Aqua shading demonstrates decreased perseverative errors during active stimulation relative to sham. Brown shading demonstrates increased perseverative errors during active stimulation relative to sham.

Discussion

This non-invasive brain stimulation study examining differences on goal-directed and habitual responding in addiction produced several notable findings, some of which was unexpected. First, we replicated our previous findings that individuals with an SUD history show an ability to execute and to learn new S-R associations (HABIT Part 1) comparable to that of controls (McKim et al., 2016a). Also replicating our previous study, we found no group differences in global performance during S-R re-learning during the HABIT Part 2, when some of the S-R contingencies changed. Moreover, we found that bilateral 10Hz-tACs stimulation of the DLPFC improved task performance after some of the S-R contingencies were changed. However, we detected a trend for a stimulation by group interaction, such that true 10Hz-tACs stimulation improved performance in the SUD group, but not the Ctrl group. 10Hz-tACs to bilateral DLPFC particularly improved the SUD group's performance in sets with unchanged S-R contingencies, whereas performance in changed sets was better during sham stimulation. These effects of stimulation were specific to very highly practiced S-R associations, and were not observed in performance of newly learned S-R associations. To our surprise, 10Hz-tACs increased perseverative errors relative to sham stimulation in the Ctrl group. Perseverative error rate was not altered by 10Hz-tACs stimulation in the SUD group, although we did observe a trend for perseverative errors to decrease more over time during true 10Hz-tACs in the SUD group. Thus, while we had predicted that 10Hz-tACs would reduce habit-based response selection and increase goal-directed response selection in people with SUDs, we instead found evidence for *increased* habitual responding among Ctrl subjects, and a much more subtle behavioral improvement in the SUD group.

Notably, the differences in behavior that we observed in this study are specific to the post-contingency change period when stimulation (true or sham) took place, ensuring minimal differences in group performance, further allowing us to demonstrate that the effects on behavior are related to stimulation condition as opposed to other, more generalized deficits. Our application of 10Hz-tACs to bilateral DLPFC showed that perseverative errors increased in the Ctrl group relative to sham stimulation; in contrast, the SUD group showed no differences in perseverative errors for either stimulation condition, although perseverative error rates did tend to decline over time in the SUD group. These results suggest that stimulation at 10 Hz disrupted the ability of control subjects to perform goal-directed responses and overcome well-learned S-R associations. These findings are surprising, given our rationale for selecting stimulation parameters in the alpha frequency range. The PFC is essential in regulating and engaging goal-directed behaviors (de Wit et al., 2009a; de Wit et al., 2012), and as a site of frontal alpha band oscillations (Klimesch et al., 2007), is a suitable target for interventions that can could result in decreases in perseverative errors. Frontal alpha activity has been suggested to increase during conditions of cognitive demand (von Stein and Sarnthein, 2000), in which attention and gating of irrelevant environmental stimuli are necessary (Klimesch et al., 2007). It has further been posited that alpha activity is necessary for top-down control, which is a function ascribed to the PFC (von Stein and Sarnthein, 2000). More specifically, the enhancing effects of tACs on entrainment of alpha oscillations in the DLPFC may be more beneficial for individuals with the SUD group relative to controls. For example, SUDs are characterized by frontostriatal circuit dysfunction (Goldstein and Volkow, 2011), and targeting the DLPFC with alpha stimulation may remediate the deficits in overcoming habitual responding that we demonstrated in our previous study (McKim et al., 2016a). Indeed, we did see lower levels of perseverative responding in the SUD group during both true and sham stimulation, suggesting

that even the 5 minutes of active sham stimulation may be beneficial in overcoming habitual responses in the SUD group. Furthermore, our results overall, and in the SUD group in particular, are likely not attributable to a placebo effect. Our analyses showed that participants were blind to the stimulation condition, and that the active sham control manipulation was effective in mimicking the sensory aspects of 10Hz-tACs, therefore confirming the differences in perseverative responding between groups as a result of stimulation.

Given our current findings, we cannot rule out that application of both 10Hz-tACs and active sham stimulation may result in better performance and enhanced goal-directed behavior in our SUD group. This change in behavior may represent a functional role for alpha oscillations in reducing frontostriatal dysfunction that is known to occur in addiction. Several lines of evidence demonstrate aberrant frontal alpha activity in addiction. Studies of resting state EEG activity in opioid-dependent patients initiating methadone treatment showed increases in local connectivity metrics in alpha and beta frequencies in fronto-central brain regions with decreases between these areas and other, more distant brain regions relative to matched controls (Fingelkurts et al., 2006). Additional evidence for alpha dysregulation during the resting state stems from studies in alcohol dependence. Research has shown decreased alpha power (Kaplan et al., 1985; Winterer et al., 2016), decreased functional connectivity in women with alcohol use disorder in the alpha band at fronto-central locations (Herrera-Diaz et al., 2016), and decreased alpha activity in recently detoxified alcoholics that increases after 6 months of abstinence (Saletu-Zyhlarz et al., 2004). Furthermore, individuals dependent on both alcohol and cocaine showed increased relative alpha power during rest (Roemer et al., 1995), which has also been found in cocaine dependent individuals (Prichep et al., 1999). Although these EEG studies were conducted during the resting state, other groups have found impairments in behaviors necessitating executive function that

correlate with alpha 'imbalance' in addiction (Davydov and Polunina, 2004; Balconi and Finocchiaro, 2015). Although speculative, our results, in combination with existing literature on alpha oscillations in addiction, suggest that alpha activity contributes to frontostriatal deficits observed in addiction. Follow-up studies that combine EEG measurement with task behavior to determine whether basal and state dependent (task-related) changes in alpha activity underlie differences in top-down and bottom-up processing are essential to answering this outstanding question.

Our finding of increased perseverative errors in control participants during 10Hz-tACs of bilateral DLPFC suggests impairment in goal-directed behavior to overcome highly practiced responses, which resulted in habitual behavior. The underlying mechanism of this behavioral impairment is unknown, although individualization of stimulation frequency to target baseline variability in the alpha frequency range may be essential to understanding our findings. Research has demonstrated that the intra-individual variability of alpha frequency can also change in a task dependent manner, such that peak frequencies can fluctuate over time (Haegens et al., 2014). Examination of our individual change plot (Fig. 4.6) demonstrates significant variability between groups, as well as within the SUD group, that may underlie the direction of our findings. For example, others have used EEG in combination with tACs to stimulate at individual alpha frequency (IAF) and have demonstrated enhancements in IAF during and for several minutes after stimulation (Zaehle et al., 2010). This study, along with our individual change data, suggest the need to determine optimal levels within the alpha band that may be more beneficial than selecting a fixed frequency. The use of tACs in combination with methods such as EEG can account for these individual differences that may impact behavior, while also measuring changing in brain activity oscillations that may result from current cognitive 'state' during task performance.

Interestingly, recent research has demonstrated that feedback controlled tACs during sleep, detecting sleep spindles and then stimulating in real time, enhanced spindle activity after tACs (Lustenberger et al., 2016); this enhancement correlated with better performance on motor memory consolidation, determined by faster reaction times for correct responses. The researchers further demonstrated that in the sham stimulation condition, the absence of any stimulation during sleep spindles, correlation between sleep spindle frequencies enhanced motor memory performance in the same frequency range in which effects were found in tACs stimulation. This most recent method of determining the effects of tACs on neural activity with real-time modulation, in combination tACs frequencies to change behavior, support the importance of targeting stimulation parameters to increase the effectiveness of behavioral effects in special populations.

We have shown that 10Hz-tACs of bilateral DLPFC, and acute stress from our study in Chapter 2, result in increases in perseverative responding in healthy control participants. The effects of stress on exacerbation of habitual responding occurs in males, but not females in the menstrual or mid-luteal phase (Chapters 2 & 3); furthermore, the ability to cope with stress, via parasympathetic modulation that we observed in males, was protective of the shift from goal-directed to habitual behavior in our task (Chapter 2). Regarding stress and arousal in our current study, the Ctrl and SUD group were matched on subjective report of perceived stress and anxiety levels. Although not significant, we did find that the SUD group showed slightly elevated levels of stress and state anxiety via self-report measures. Individuals with higher levels of anxiety, indexed with self-report on the STAI (questionnaire we also used), have been found to show positive correlations between this subjective measure and physiological reactions to stress (Bali and Jaggi, 2015). We did not collect biological measures of stress via heart rate or cortisol in this study, but we did not find correlations between anxiety levels or stress and perseverative

responding in either group. While these findings are not causal, they support the notion that these factors, as proxies of stress, did not impact our results. However, given the novelty of our brain stimulation manipulation, and potential anticipatory effects of this minimally invasive study manipulation, it may have affected aspects of performance that we did not assess biologically. This further emphasizes the necessity of an 'active' sham condition in which neurosensory affects and potential anticipatory arousal would occur during this study session. Taken together, the evidence from our study suggest that the effects of stimulation and stress on behavior are likely to occur independently. Future studies that directly measure stress or arousal mechanisms prior to or during stimulation, which are presumably acting via modulation of PFC function, are needed to determine whether the effects of stress on behavior are occurring independently or in combination with stimulation.

Findings from our study suggest that the behavioral effects of tACs may also follow an inverted-U model, in line with stress and arousal (Yerkes and Dodson, 1908), as well as working memory models (Sawaguchi and Goldman-Rakic, 1991), of task performance. This hypothesis is supported by the tailoring of stimulation to the IAF (Zaehle et al., 2010), and that state dependent shifts can occur on what is an 'optimal' level within an individual during task performance time (Haegens et al., 2014). Our observations of individual variability in perseverative behavior between the stimulation conditions may relate to suboptimal enhancement of alpha in the SUD group, or excess alpha activity that may have shifted behavior more dramatically in the Ctrl group. Our results provide preliminary data for future studies to probe the underlying neural bases of the changes we found at the behavioral level with 10Hz-tACs of bilateral DLPFC. The development of novel methods for the application of tACs, including real-time feedback methods, will have

wide therapeutic applications for disorders characterized by aberrant neural oscillation frequencies, including addiction and others such as schizophrenia.

CHAPTER 5

GENERAL DISCUSSION

Studies thus far investigating the behavioral and neural correlates of goal-directed and habitual behavior have largely used paradigms that promote habitual responding in the lab on a shorter timescale compared to ingrained behaviors that develop in the real world. Unlike other task paradigms, our HABIT paradigm includes training and a subsequent test session, which can differentiate between highly practiced versus freshly learned S-R associations; multiple permutations of the task also enable measurement of the stability of behavioral and neural correlates over time (Boettiger et al., 2004). This aspect of our task can better capture the timescale of the development of entrenched, and hard to change, S-R behaviors that mimic the development of habits outside of a laboratory setting. Furthermore, our task includes a novel response devaluation manipulation, which allows us to quantify behavioral responses during attempts to break habitual responding. The habit breaking process has not been extensively investigated in human studies of addiction, and our model lends itself to examine this avenue of research to develop novel therapeutic interventions for addiction.

These advantages of our HABIT paradigm, in combination with our previous data showing increased habit-based responding in individuals with an SUD history relative to control subjects (McKim et al., 2016a), highlight the utility of testing manipulations or interventions that can potentiate habitual responding or diminish habit based responding, respectively. Thus, the overarching goals of the present dissertation were to investigate the role of psychosocial stress in

shifting behavior toward habit-based responding in healthy controls, and to test the use of non-invasive brain stimulation to reduce habit-based responding in individuals with an SUD history. Using our HABIT paradigm in combination with these biological interventions, we demonstrate that stress increases perseverative responding, our index of habitual behavior, in healthy males; moreover, this effect depends on the context of stress, and biological activation of the parasympathetic nervous system protects against stress-based effects. In contrast, we found gender differences such that females do not show increases in perseverative errors after either stress context manipulation, which likely reflects protective effects of ovarian hormones against this shift in behavior from goal-directed to habitual. Finally, we demonstrate that 10Hz-tACs of bilateral DLPFC, relative to active sham stimulation, increased perseverative responding in healthy controls, while true stimulation relative to sham stimulation showed no clear effect on perseverative errors in the SUD group. Together, these data extend the current literature by not only demonstrating the utility of our task paradigm to measure goal-directed and habitual behavior across studies and cohorts of healthy controls and individuals with SUDs, but also provides evidence regarding mechanisms for modifying goal-directed and habitual behavior. Our findings provide a foundation for future study of pharmacological interventions to block stress effects on shifts in behavioral responding, as well as demonstrate the need for further investigations into the brain rhythms, in addition to alpha oscillations, that may underlie excessive habitual responding in addiction. The studies in this dissertation also have broader implications for other disorders, such as OCD, that may be characterized by well-established patterns of behavior that are difficult to change.

Summary of Experimental Findings

To investigate the effects of acute psychosocial stress on goal-directed and habitual responding, we examined whether behavior within our HABIT paradigm would be dependent upon stress context in healthy males. We found that stress prior to the HABIT Test Part 1 selectively increased perseverative responding, our main index of habitual behavior, after response devaluation; importantly, stress did not disrupt goal-directed learning prior to response devaluation in this group of males. Additionally, we found similar levels of salivary cortisol increases among our male stress groups, demonstrating robust activation of the HPA axis in response to acute psychosocial stress. However, we found that males within the stress before HABIT Test Part 2 study group had enhanced parasympathetic control in terms of HR and HRV metrics during and after stress. The biological regulation of the effects of stress in this manner suggest a protective effect on the stress induced shift toward habitual responding that we found in the other group of stressed males. Moreover, analyses of the relationship between HPA axis activation via salivary cortisol, based on cortisol responder status and correlation between peak cortisol levels with perseverative errors, demonstrated the independence of the habit-based behavioral effect from cortisol responsiveness. In accordance with these findings, we hypothesize that the stress-induced increase in habitual responding in males stressed before HABIT Test Part 1 reflects strengthening or 'stamping in' of the habitual behavior, which resulted in deficits in changing this highly trained behavior after response devaluation. Parasympathetic modulation of the effects of acute stress are preventive in the switch toward habitual responding, presumably through top-down cognitive regulation to flexibly use differential response strategies during learning and re-learning.

In an effort to better characterize the impact of stress timing on habitual behavior in healthy females, we next sought to examine two phases within the menstrual cycle when ovarian hormones

are relatively static but lower or higher overall, on goal-directed and habitual responding. We collected data from females during two phases of the menstrual cycle: the menses phase (MP group) and the luteal phase (LP group). During these phases of the cycle, ovarian hormone levels of estrogen and progesterone are relatively static, with lower levels of estrogen and progesterone during the menses phase relative to elevated levels of both during the luteal phase. We found that females using hormonal birth control, and females within the LP group of our sample, showed better performance on goal-directed learning in HABIT Test Part 1. Interestingly, we did not find an increase in perseverative responses as a result of stress, as we demonstrated in males. Similar to our results in males, we did find that perseverative errors in females were independent of cortisol responsiveness. When examining heart rate data in females, we found increased sympathetic activation during stress, regardless of menstrual cycle phase. However, within the MP group, there was a negative correlation between increased HR during stress and perseverative responding. Since ovarian hormones are static and lower during this phase, it suggests that in these females, stress actually had the opposite effect to males; stress was associated with increases in goal-directed control over habitual responding, resulting in reductions of perseverative errors after stress for females within this cycle phase.

We specifically did not target ovulation during the follicular phase due to a surge in estrogen that has been shown to impact PFC dopamine-dependent behaviors in an inverted-U shaped manner (Jacobs and D'Esposito, 2011; Smith et al., 2014); moreover, stress has been shown to have effects on cognition and behavior according to an inverted-U-shaped function (Yerkes and Dodson, 1908). These lines of evidence, along with previous neuroimaging results in healthy controls using our HABIT paradigm that demonstrated increased goal-directed learning associated with activation of the DLPFC (Boettiger and D'Esposito, 2005), suggest that prefrontal function is

critical to task performance in general, and goal-directed behavior specifically. Evidence from rodents and nonhuman primates has consistently demonstrated that stress increases levels of both dopamine and norepinephrine within the PFC, altering the architecture of neurons at the subcellular level, and ultimately leading to weakened synaptic connections and diminished cognitive control (Arnsten, 2009, 2015). In contrast, subcortical connections between the striatum and amygdala are strengthened, and can lead to automatic behavior, without PFC top-down control, under conditions of stress (Arnsten, 2015). Our behavioral results in females, relative to our male findings of an increase in perseverative responding after stress prior to HABIT Test Part 1, suggest that stress is not detrimental to goal-directed performance during these menstrual cycle phases within our sample. As we did not directly measure ovarian hormone levels, it is also plausible that there may be synergistic effects of stress and ovarian hormones on behavior, via evidence that their common mechanism of action is within the PFC. Future studies that account for menstrual cycle phase in females, and that include direct measurement of ovarian hormone levels, are needed to examine the neural correlates of goal-directed and habitual behavior to determine whether the mechanism of action is separate, or could be synergistic, within the PFC.

The final chapter of this dissertation (Chapter 4) tested the effects of tACs on reducing the high rates of perseverative responding we previously found in people with an SUD history (McKim et al., 2016a). Using the same paradigm, we tested a new cohort of SUD history individuals and controls. We again demonstrated that our SUD sample were not impaired in the ability to execute or learn new S-R associations relative to controls; furthermore, there were not global deficits during S-R re-learning in our SUD group. These findings replicate our previous published data (McKim et al., 2016a). Bilateral 10Hz-tACs of the DLPFC improved performance for both groups during the re-learning task phase (HABIT Test Part 2), and we observed a trend

for this to differ between groups. The SUD group was likely to show improved performance relative to the control group during true stimulation. The effects on performance were specific to highly practiced versus newly introduced S-R sets. Surprisingly, when we examined our main index of habitual responding, perseverative errors, we found increased perseverative errors in the Ctrl group during true stimulation. In the SUD group, true stimulation showed a trend toward decreasing perseverative errors over time, but it did not decrease the overall amount of perseverative errors during re-learning. Despite our findings being in the opposite direction of what we predicted, our data provide a foundation for future studies with our paradigm to test other stimulation frequencies and incorporate more individualized manipulations of neural oscillations. The extensively developed PFC in humans, as the seat of executive control (Fuster, 2008), is a prime target for use of novel brain stimulation techniques to perturb or enhance ongoing activation and test effects on cognition, and ultimately behavior. A combination of methods may be best suited to further probe these behavioral effects, in addition to more widely used techniques such as EEG, fMRI, and TMS; the use of transcranial electrical stimulation (tDCs, tACs), feedback controlled stimulation protocols, multiple coil TMS, or state dependent studies of cognition and behavior are on the horizon (Romei et al.; Lustenberger et al., 2016). These methods will better our understanding of the functional role of brain regions or networks in behaviors of interest, not only in normal functioning, but in mental health disorders that are characterized by neural network dysfunction.

Neural Circuitry Involved in Goal-Directed and Habitual Behavior

Stimulus-response learning allows us to respond efficiently to familiar stimuli in our environment, while cognitive flexibility allows us to modify automatic responses when action-

outcome contingencies change. Goal-directed and habit-based behaviors each rely on distinct frontostriatal circuits, according to several lines of data (de Wit et al., 2007; Kalivas, 2008; Kehagia et al., 2010; Noonan et al., 2011; Hadj-Bouziane et al., 2013). For example, lesion studies in both animals and human patients show the importance of frontostriatal circuits in the transition from goal-directed to S-R behaviors (Petrides, 1982, 1985, 1997; Murray et al., 2000; Naneix et al., 2009; Stalnaker et al., 2010). Moreover, rodent studies demonstrate the role of connections between the putative rodent homolog of the DLPFC, the medial prefrontal cortex (mPFC) (Farovik et al., 2008), and distinct striatal subregions during goal-directed versus habitual behavior (Coutureau and Killcross, 2003; Killcross and Coutureau, 2003; Yin and Knowlton, 2004; Yin et al., 2004, 2005a; Yin et al., 2005b; Yin et al., 2006b; Yin et al., 2006a; Yin et al., 2008; Naneix et al., 2009; Tran-Tu-Yen et al., 2009; Stalnaker et al., 2010; Izquierdo and Jentsch, 2012; Smith et al., 2012a; Rhodes and Murray, 2013; Smith and Graybiel, 2013). Studies in primates, as well as human neuroimaging studies, complement these findings by demonstrating roles for the DLPFC in the goal-directed formation of novel S-R associations and for the dorsal striatum in mediating habitual behavior (Asaad et al., 1998, 2000; Toni et al., 2001; Wallis et al., 2001; Wallis and Miller, 2003; Boettiger and D'Esposito, 2005; Pasupathy and Miller, 2005; Muhammad et al., 2006; Fusi et al., 2007; Valentin et al., 2007; Loh et al., 2008; de Wit et al., 2009b; Tricomi et al., 2009; Hiebert, 2014). The brain areas studied above are within the same neural circuits that have been found to be abnormal in addiction (Koob and Volkow, 2010; Goldstein and Volkow, 2011), suggesting that dysfunction within frontostriatal circuits may shift the balance between goal-directed and habitual action selection (Everitt and Robbins, 2016).

In SUD populations, stress is a potent trigger of relapse, but the neurobiological basis is unknown (Sinha, 2009, 2012). Several lines of evidence suggest that stress can alter the neural

circuits underlying goal-directed and habit-based responding, which could theoretically drive continued and maladaptive drug use after relapse. Alterations in frontostriatal neural control over these behaviors have been demonstrated during stress in healthy controls that are comparable to deficits at the neural level from fMRI data in persons with SUDs (Goldstein and Volkow, 2011; Schwabe et al., 2011a). A neuroimaging study in combination with pharmacological administration of hydrocortisone, yohimbine, or both to induce biological stress responses prior to instrumental training, demonstrated a disruption of outcome value processing in brain areas such as the mPFC and OFC, which rendered behavioral habitual (Schwabe et al., 2012). An examination of more 'naturally' occurring stress outside of the laboratory setting was tested by (Soares et al., 2012) in students preparing to take a medical examination. Using the same instrumental learning task as the Schwabe group, participants were tested prior to taking the exam in a stressed state, and again 6-7 weeks after the exam in a non-stressed state. Results demonstrated reversible effects of chronic stress on behavior that changed from more habit-based prior to stress, to more goal-directed when tested again in a non-stressed state. At the neural level, there were volumetric changes with prefrontal and striatal brain regions necessary for these behaviors that also normalized over time from pre- to post-stress. These studies demonstrate that changes within both prefrontal and striatal brain regions, dependent upon stress type and duration, underlie the shift in behavior from goal-directed to habit-based.

To begin to integrate the neuroimaging findings of a stress-induced shift toward habitual behavior, and our previous findings that SUD history individuals showed a heightened propensity to form habits (McKim et al., 2016a), we manipulated stress in healthy controls with our HABIT paradigm. Our findings showed that acute stress potentiated habitual responding in healthy adult males stressed prior to practice of learned S-R sets. This effect was specific to well-learned S-R

actions, and stress occurring prior to acquisition of newly introduced S-R sets did not inhibit S-R learning, which heavily recruits DLPFC (Boettiger and D'Esposito, 2005). Together, our findings suggest that changes at the neural level underlying enhanced habitual responding include a shift toward recruitment of striatal circuitry without deficits in prefrontal control. This conclusion is in line with neuroimaging results from chronic stress effects on behavior and changes in striatal volume, but differs from acute, pharmacologically induced stress, which resulted in deficits in prefrontal control over behavior. These differences may stem from differential activation of the HPA axis and sympathetic nervous system under conditions of acute versus chronic stress. Furthermore, training and test within a single study session may not have fully engaged habit-based neural circuitry relative to the extended amount of execution of S-R sets in our HABIT paradigm that can promote inflexible responding.

Our novel behavioral findings are supported by recent research in animals on the role of the striatum in action initiation, execution, and habit-based responding. The canonical model of basal ganglia function postulates that two distinct circuits originating in the striatum, the direct (striatonigral) and indirect (striatopallidal) pathways, project to different output structures, which result in opposite effects on movement (Calabresi et al., 2014). This model suggests that function of the direct pathway is likely to promote movement, while activation of the indirect pathway inhibits movement. However, recent research has challenged this model, showing that both pathways are active during action initiation and performance of instrumental choice behavior (Cui et al., 2013; Tecuapetla et al., 2016). These findings demonstrate that the striatal pathways regulate movement in a complementary manner, with the relative activation of these brain regions during behavior predicting habitual responding. Interestingly, direct pathway projection neurons in the DLS were shown to be active prior to indirect pathway neurons in mice that were insensitive to

outcome devaluation (O'Hare et al., 2016). The researchers further tested habit suppression with a reward omission test after extended training, demonstrating that a 'reversal' or decrease in well-trained responding was associated with decreased output signals in the direct pathway; however, this behavioral suppression effect was not related to indirect pathway output activity (O'Hare et al., 2016). Although these studies do not account for specific cortical input into the striatum, the results demonstrate that local processing and integration within the DLS is sufficient to predict expression of habitual behavior. The enhanced processing mechanisms within this area of the rodent striatum, the human equivalent of the putamen, may underlie the shift to increased perseverative responding that we found in males. Our results add further support to the growing body of literature demonstrating that stress facilitates bottom-up processing at the expense of the PFC to regulate top-down control. Moreover, the location of the striatum makes it an essential connection hub to regulate input-output activity for instrumental choice behavior.

Similar to results of concurrent activity changes within the striatal direct and indirect pathways, recent evidence also suggests coordinated connections between the prefrontal cortex and striatum contribute to control of goal-directed and habitual behaviors. Studies in mice have demonstrated that neurons within the OFC and DMS shift activity to be more engaged during the use of a goal-directed strategy relative to their activity levels during habit-based responding (Gremel and Costa, 2013); the OFC and DMS also showed increased activation, relative to DLS activation levels, in animals using a goal-directed strategy relative to habitual strategy after reward devaluation. Importantly, these data demonstrate that neuron ensemble activity can vary along the continuum of behavior, further supporting the idea that the same neural circuits are active during both types of behavioral responding, and that their relative contribution to circuit level activation can predict which behavioral strategy is utilized. These findings further challenge the original

hypothesis that activity shifts between neural circuits mediating goal-directed and habit-based behavior, instead demonstrating that activity within these neural circuits can change over time with behavioral flexibility.

Research in humans has also begun to focus on the dynamic interplay between prefrontal and striatal brain regions during learning and execution of instrumental choice. Neuroimaging studies have demonstrated that several prefrontal brain areas act in concert to during goal-directed behavior, including the DLPFC, vmPFC/OFC, and ACC (Dolan and Dayan, 2013), with the recent suggestion that prefrontal areas can arbitrate between response selection strategies (Lee et al., 2014); this suggests that relative to animal work, a wide variety of PFC brain areas could contribute to goal-directed behavior that is tested within the lab. Furthermore, human studies of the OFC complement results from animals that demonstrate the importance of the OFC in goal-directed behavior. In humans, learning to generate a new response to a familiar stimulus, essentially "overwriting" an existing S-R association appears to depend on the OFC (Boettiger et al., 2004). Evidence to support this role of the OFC also stem from reversal learning studies in animals (Dias et al., 1996; McAlonan and Brown, 2003; Schoenbaum and Setlow, 2005; Ostlund and Balleine, 2007; Schoenbaum et al., 2007; Tait and Brown, 2007), with additional evidence in human neuroimaging studies demonstrating that overcoming learned response contingencies involves the OFC (Elliott et al., 2000; Cools et al., 2002; Boettiger et al., 2004; O'Doherty et al., 2004; Remijnse et al., 2006). The variety of prefrontal brain areas that contribute to goal-directed behavior, in combination with recent animal work focused on the role of OFC on goal-directed behavior, suggest that our behavioral findings of increased perseverative errors in healthy controls during bilateral tACs stimulation of the DLPFC may reflect a disruption in communication between multiple prefrontal brain regions that resulted in striatal control of response selection.

The extensively developed prefrontal cortex in humans, comprising several brain areas with varying functions, supports executive function. These brain regions together coordinate behavior toward the ultimate goal of action selection. For example, extensive study of the DLPFC has demonstrated that it is essential for working memory, which underlies temporary manipulation and storage of information (Fuster, 2008). In contrast, the OFC has been shown to compute outcome value and display prediction error signals related to expected versus received reward (Balleine and O'Doherty, 2010; O'Doherty, 2011). While both of these brain regions have been shown to be necessary for goal-directed behavior, previous neuroimaging data from our HABIT paradigm in healthy controls showed that changing well-established S-R associations relied on recruitment of the OFC in combination with the ACC (Boettiger et al., 2004), which functions to detect conflict and monitor response errors. Combined activation of the OFC and ACC suggests that successful change of response mappings for highly practiced action sequences requires coordinated activity of multiple prefrontal areas; in contrast, DLPFC activation predominates during initial goal-directed learning of S-R associations (Boettiger and D'Esposito, 2005). It is therefore plausible that our use of bilateral DLPFC stimulation also disrupted function within the OFC and ACC, which resulted in increased perseverative responding. This behavioral result may reflect an inability to register the change in outcome value or to use feedback to adapt well-practiced S-R associations after response devaluation. Furthermore, all of these prefrontal brain areas have been shown to converge within the rostral, dorsal caudate nucleus, an area essential for goal-directed action selection (Choi et al., 2016). These functional connections were most likely disrupted by tACs stimulation of bilateral DLPFC, suggesting changes in circuit level oscillatory dynamics between these brain regions relative to their baseline state. Our behavioral finding of increased perseverative errors in healthy controls during true stimulation further suggests that these

automatic response tendencies were a result of putamen activation and reflexive action selection that occurred during altered corticostriatal circuitry underlying goal-directed control.

Results from our studies identify two manipulations that can increase habitual responding, which can be further investigated to clarify their neurobiological mechanisms of action. The effects of acute stress and tACs of bilateral DLPFC likely shift behavior through enhanced activation of the putamen, the major brain region implicated in habit-based responding. Our behavioral findings reiterate the importance of the striatum as a connection hub between prefrontal input and striatal output pathways on response selection. Furthermore, previous research in humans and animal models demonstrating the dynamic interplay between corticostriatal circuitry, in combination with our noninvasive brain stimulation results here, highlight the importance of neural circuit balance. A better understanding of neural circuit communication, specifically the brain oscillation frequencies underlying goal-directed and habitual behavior in both normal and abnormal conditions, is needed. Such findings will ultimately inform novel approaches for treatment of disorders that may be characterized by compromised prefrontal function.

Future Directions

Several notable findings were obtained through the current set of experiments, and contribute to our understanding of goal-directed and habitual action selection. We demonstrated that acute stress does not impair the acquisition of S-R associations, but that acute stress can promote habitual responding in healthy males. We found that the shift in behavior toward habitual responding was linked to sympathetic activation relative to HPA axis activity; males that showed enhanced parasympathetic modulation of heart rate during stress were protected against the shift toward habit behavior. Parasympathetic control may have arisen in this male stress group due to

task performance requiring top-down control for goal-directed learning of S-R sets, as well as switching between previously learned and newly acquired S-R associations. This engagement of prefrontal brain areas may have buffered the detrimental biological effects of the stress response by activating areas such as the mPFC that has been shown to regulate control over, and resilience to, stress (Thayer et al., 2009; Thayer et al., 2012). Taken together, these results suggest that activation of the sympathetic and parasympathetic nervous system are the underlying biological mechanism of enhanced or reduced perseverative errors, respectively. As such, a study to pharmacologically block this stress-induced shift in behavior, using the beta-adrenergic antagonist, propranolol, could further strengthen and confirm our behavioral findings. This manipulation would clearly delineate the biological link between increased perseverative errors that we observed in males and sympathetic activation, relative to contributions of the HPA axis during stress. This mechanism could be tested in both control individuals and those with an SUD history to further probe whether increased perseverative errors in the SUD group that we found previously were due to stress. Alternatively, another way to prevent the stress response may be through parasympathetic regulation of heart rate, including techniques such as mindfulness mediation or biofeedback based on HF-HRV (de Bruin et al., 2016). These interventions are designed to increase communication between the brain and the body through mechanisms of conscious or top down control; this is similar to our suggested mechanism of action of parasympathetic regulation in our male group that was engaging in top-down control during task performance prior to stress. These behavioral interventions may be more readily available to implement in a clinical treatment setting since they do not involve direct pharmacological intervention, and as such, they may be a low cost alternative. Moreover, a wider population of individuals may benefit as a result of less stringent recruitment criteria, e.g. contraindications to medication, as well as eliminating the issue of compliance to a

regular medication schedule.

In contrast to the relationship between NS activation and an increase in perseverative errors that we found in males, we did not find a clear result for a biological effect of stress on behavior in females. This effect was not due to an absence of a biological stress response, as females showed elevations in HPA axis activity and heart rate indices of sympathetic activation; rather, it could reflect differences in psychological processing of the stress context. Perhaps measurement of behavioral coping strategies during or after stress via self-report or behavioral observation could provide insight into gender differences in reaction to the stressor. These metrics could determine whether women were more likely to displayed 'tend-and-befriend' behaviors as opposed to sympathetic activation that is related to the fight or flight response commonly shown in males (Taylor et al., 2000). Future studies could incorporate video recording of behaviors during and after stress to determine whether women seek social interaction or affiliative behaviors to manage changes in mood or anxiety related to stress.

Further investigations into the relationship between ovarian hormone levels to determine whether estrogen, as well as progesterone, could underlie psychological and biological responses to stress are needed. To begin to address this gap in the literature, females could be recruited during the follicular phase near the ovulation window, when estrogen is at its peak. During this point in the cycle, prefrontal dopamine levels will also likely be higher, suggesting a potential benefit to performance on goal-directed learning. This would also most likely depend on catechol-O-methyltransferase (COMT) genotype, which is a putative genetic marker of levels of PFC dopamine (Chen et al., 2004). Females with met/met genotype, having lower COMT enzyme activity, and thus higher levels of prefrontal dopamine, may perform worse and perseverate, if combined with a surge in catecholamines from stress. In contrast, val/val females, having higher

COMT enzyme activity and lower levels of PFC dopamine, may actually perform better at the task overall, or when combined with stress, likely have better performance relative to met/met females (Jacobs and D'Esposito, 2011). This relationship between performance, PFC dopamine, and estrogen likely follows an inverted-U-shaped model, which has been demonstrated by other female cycle studies of PFC dependent behavior (Jacobs and D'Esposito, 2011; Smith et al., 2014). Furthermore, a necessary control in these studies would be the collection of saliva or blood sample(s) to confirm cycle phase in addition to self-report, as well as be able to test for individual variability in levels of hormones across the cycle; a within subjects design could be employed to test whether behavioral changes are evident within the menstrual cycle to determine whether they are related to the fluctuations in ovarian hormones.

Given the importance of prefrontal function for working memory during goal-directed action selection, we tested whether bilateral alpha band tACs of the DLPFC would reduce perseverative responding in individuals with an SUD history. Surprisingly, we found that true stimulation increased perseverative errors in the control group relative to sham stimulation; in contrast, we did not find a decrease in perseverative errors as expected in the SUD group during true stimulation. Given these results, several outstanding questions remain on the underlying mechanism of action of the stimulation. Since we targeted a fixed frequency within the alpha band (10Hz) it would be beneficial to know how this relates to an individual's baseline or 'normal' EEG pattern of alpha activity in the DLPFC. Future studies that incorporate EEG metrics may allow an individualized target frequency to be selected per individual, and if measured during task performance prior to stimulation, would provide empirical data on whether the individual frequency of activity within the PFC changes over time or is state dependent (Haegens et al., 2014); these metrics may improve the benefits of the stimulation on task performance. Although there is

evidence in the literature that alpha stimulation may be dysregulated in addiction (Davydov and Polunina, 2004; Balconi and Finocchiaro, 2015), along with alpha playing a role in top-down control in healthy individuals (Klimesch et al., 2007), another important experiment would be to include a different stimulation frequency, such as theta, to determine whether modulation of behavior is frequency specific. Theta has been implicated in working memory studies, as well as reversal learning, and as such, would be a good alternative to test in our HABIT paradigm (Hsieh and Ranganath, 2014; Jausovec and Jausovec, 2014; Wischnewski et al., 2016). Furthermore, relating back to our studies of stress, theta activity has been shown to be decreased under acute stress (Gartner et al., 2014), and could represent an important frequency to target to better understand the relationship between stress, working memory, and performance of goal-directed and habitual action selection strategies. Taken together, the findings from the studies discussed within this dissertation, along with the open questions that can be answered by the proposed experiments detailed here, demonstrate the necessity of studying goal-directed and habitual behaviors in addiction, as well as daily life. Behaviors that we perform on a daily basis require the use of both goal-directed and habitual actions, highlighting the importance of intact communication between neural circuits. Future studies that aim to disentangle the role of aberrant neural function, including investigating into the role of specific oscillation frequencies, is a promising avenue for novel treatments for SUDs, as well as many other mental disorders.

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