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Examining the Roles of Sex, Methamphetamine, and Degree of Training in Habit Formation in Rats

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EXAMINING THE ROLES OF SEX, METHAMPHETAMINE, AND DEGREE OF TRAINING IN HABIT FORMATION IN RATS

A Thesis Presented

by

Hannah L. Schoenberg

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Master of Arts Specializing in Psychology

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ABSTRACT

Addiction is characterized by a progressive loss of executive control over drug-seeking and consumption, and may be associated with a behavioral shift from instrumental goal-directed actions to stimulus-response habits. Sex differences in drug addiction have been linked to changing hormone levels across the estrous cycle, and females exhibit a particular vulnerability to psychostimulants such as cocaine and amphetamines. Psychostimulants and estrogen both influence dopaminergic activity in the dorsal striatum, a region of the brain in which dopamine activity is thought to mediate the shift from action to habit. In the present set of experiments, we examined the roles of sex, amphetamine, and degree of training on habit formation in rats. To test habit formation in each experiment, animals were trained on a variable interval (VI) schedule of reinforcement to nose-poke for sucrose pellet reinforcers, then the sucrose was devalued in half of the animals by pairing its presentation with injections of lithium chloride (LiCl) to induce nausea. Animals for whom the sucrose was paired with LiCl acquired a conditioned taste aversion for the sucrose reinforcer. When tested in extinction, paired animals who remained goal-directed should inhibit their responding for the devalued sucrose, whereas animals in habit should be insensitive to the devaluation and respond at a similar rate as their non-devalued counterparts.

Experiment 1 examined the role of sex in habit formation in which intact male and female rats received identical training, devaluation, and testing in two separate within-sex experiments. After 240 reinforcer exposures females exhibited habitual behavior whereas males remained goal-directed. In Experiment 2, female rats were ovariectomized (OVX) and half were given cyclic estrogen replacement. All animals received either pre-exposure to methamphetamine (METH) or vehicle. Following exposure to 120 reinforcers, a level of training that had previously been shown to be subthreshold to habit formation in males, all female groups demonstrated goal-directed responding at test, revealing a lack of effect of hormone replacement or drug pre-treatment on habit formation in OVX females at this level of reinforcer exposure. Experiment 3 aimed to determine the degree of nose-poke training that would be subthreshold to habit formation in intact females, and two groups were given different amounts of training. Both groups exhibited habitual responding, indicating that habit threshold in females is lower than hypothesized. Overall, these experiments suggest that females shift into habitual behavior earlier in training than males, and further experiments need to be conducted to determine how factors such as hormone milieu and psychostimulant exposure influence this progression.
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CHAPTER 1. LITERATURE REVIEW

1.1 Characterizing Actions and Habits

The development of habitual behaviors is a well-documented behavioral phenomenon. In the early stages of learning, instrumental behaviors are goal-directed, action-outcome (A-O) processes, wherein the performance of a behavior is driven by the responders’ knowledge of the outcome of the behavior; consequently, the performance of these goal-driven behaviors are sensitive to changes in the value of the outcome (Adams & Dickinson, 1981). In later stages of learning, associative stimulus-response (S-R) processes can become dominant (Dickinson, 1985). Under S-R learning, the power of the outcome to drive the behavior is diminished, and instead the behavioral response is initiated reflexively in the presence of stimuli that have become associated with the outcome. Therefore, S-R behaviors are insensitive to changes in outcome value (Adams, 1982). The automaticity with which responses are made in S-R learning results in the consideration of these behaviors as habits.

Habitual behaviors can be adaptive by promoting behavioral efficiency and increasing the availability of cognitive resources (Lingawi, Dezfooli, & Balleine, 2016), however, they may also become maladaptive. Responses that are divorced from their outcome can become problematic when the consequences of the behavior become pathological. This is evident in the continuation of fear responding, despite the presence of a safety signal, seen in many anxiety-related psychopathologies, such as PTSD (Goodman, Leong, & Packard, 2012), in the inability to control motor behavior in obsessive-compulsive disorder (Goodman et al., 2012; Ferreira, Yücel, Dawson,
Lorenzetti, & Fontenelle, 2017) and when maladaptive incentives towards drugs of abuse elicit habitual responding in addiction (Belin, Belin-Rauscent, Murray, & Everitt, 2013).

Assessing Habitual Responding by Devaluing the Outcome

As discussed above, goal-directed behavior is sensitive to changes in the value of the outcome, whereas habitual behavior is not. Therefore, a common method for testing if behavior is goal-directed or habitual is to devalue the outcome, then measure responding under extinction conditions (Adams, 1982; Adams & Dickinson, 1981). When A-O processes are dominant, devaluing the outcome should lead to a reduction in responding, as the action is associated with the outcome that is no longer motivating. However, this reduction should not be observed when behavior is habitual, as responses are elicited from associated stimuli and these associations (S-R) do not monitor the current value of the outcome. There are two devaluation procedures: specific satiety (e.g. Balleine & Dickinson, 1998a) and conditioned taste aversion using lithium chloride (LiCl, e.g. Adams, 1982). In rodents, specific satiety is generally accomplished by allowing the animal free access to the food reinforcer they earned during training (e.g. grain or sucrose pellets) prior to testing for habit under extinction conditions. Animals who are sated will not be motivated to perform the action required to earn the now-devalued reinforcer if they are still operating under A-O processes. In a conditioned taste aversion paradigm for reward devaluation (RD), after training occurs, half of the animals are delivered the reinforcer earned during training paired with an injection of LiCl, which induces nausea, and half receive the reinforcer unpaired with LiCl (non-contingently). In the paired group, a taste aversion is conditioned such that animals learn that the reinforcer leads to
illness, thereby reducing its hedonic value. Paired animals that remain goal-directed should then demonstrate lowered responding at test than unpaired animals, whereas paired animals for whom a habit has been trained should exhibit no such reduction.

**Correlation Between Reward Rate and Response Rate**

The factors that determine the transition from action to habit are an ongoing topic of investigation. From a behavioral perspective, there is evidence suggesting that rather than simple repeated exposure to the action-outcome contingency, strength of the correlation between the response rate and reward rate dictates the dominant action control process. A strong correlation strengthens the A-O association favoring goal-directed actions, and a weaker correlation allows for S-R responding to become dominant (Dickinson, 1985). The correlation can be modulated both by the total amount of training (number of action-outcome contingencies), as well as the schedule of reinforcement on which the learning or training occurs. Dickinson (1985) observed that at the onset of training response rates are more varied; therefore, there is a wider range of response rates under which the animal experiences the reward rate, leading to a stronger correlation. Later in training, response rates asymptote, resulting in a limited range of response rates under which the reward rate is experienced, and subsequently to a weaker correlation between response and reward rate. Consequently, as training progresses, the correlation between response and reward rate weakens. This is supported by extensive evidence that with limited training, behavior tends to be goal-directed whereas extended training results in habitual behavior (Adams, 1982; Dickinson, Balleine, Watt, Gonzalez, & Boakes, 1995; Balleine & Dickinson, 1998b; Coutureau & Killcross, 2003).
Schedules of reinforcement also play a role in the strength of the correlation between reward and response rates. Under ratio schedules, reinforcers are delivered after a certain number of responses are made; since response rate directly relates to reward rate under this schedule, goal-directed behavior endures. Under interval schedules, reinforcers are delivered based on certain amounts of time passing before responses are rewarded, therefore response rate and reward rate are not as tightly related. Experiments using both schedules of reinforcement with equivalent amounts of training have demonstrated that indeed, ratio schedules of reinforcement lead to ongoing goal-directed behavior, whereas interval schedules of reinforcement are associated with habit formation (Dickinson, Nicholas, & Adams, 1983).

1.2 Neural Correlates of Actions and Habits

A full, in-depth review of the circuitries that mediate the acquisition, expression, and maintenance of actions and habits is beyond the scope of this review (for full review, see Balleine & O’Doherty, 2010; Belin et al., 2013). Here, we will briefly discuss principle brain structures involved in these behaviors and focus primarily on the contribution of the striatum, particularly the dorsal striatum, in the selection and performance of actions and habits. The dorsal striatum is subdivided into two functionally heterogeneous regions: the dorsomedial (DMS) and dorsolateral (DLS) striatum, which are thought to mediate goal-directed and habitual behavior, respectively, via action selection and motor output.
**Goal-Directed Behavior**

As mentioned previously, the expression of goal-directed behavior is driven by knowledge of the value of the outcome; therefore the DMS, which mediates this behavior, must receive information regarding the hedonic value of the outcome, or reinforcer. One proposed mechanism for this process is that the basolateral amygdala (BLA) associatively links the sensory properties of a reinforcer with its incentive value (Balleine & O’Doherty, 2010), and that this coded information is then relayed to the nucleus accumbens core (NacC) which influences dopaminergic innervation of the DMS where action selection and subsequent motor output is mediated. Cortical input to goal-directed action selection is thought to come from the prelimbic cortex (PL), which exerts top-down control over the BLA and NacC, as well as projects directly to the DMS (Belin et al., 2013). Studies in which the PL and DMS were lesioned before or after behavioral acquisition have demonstrated that the PL is necessary for the learning of goal-directed behavior, but not for its behavioral expression (Ostlund & Balleine, 2005), whereas both learning and expression requires DMS involvement (Yin, Ostlund, Knowlton, & Balleine, 2005; Gremel & Costa, 2013; Corbit & Janak, 2010).

**Habitual Behavior**

Similar lesion studies have been employed to study the role of the DLS in habitual behaviors: following overtraining (to the point where behavior is dominated by S-R processes), lesioning the DLS restores sensitivity to the outcome of instrumental actions (Zapata, Minney, & Shippenberg, 2010; Gremel & Costa, 2013). Additionally, lesioning the DLS prior to overtraining prevents the acquisition of habitual behavior (Yin,
Like the DMS, the DLS is required for both the acquisition and expression of habitual responding. In particular, dopaminergic innervation of the DLS is necessary for habit formation: lesions of the nigrostriatal dopamine pathway, blocking this innervation, also prevents habit formation (Faure, 2005).

The infralimbic cortex (IL), a subregion of the rodent prefrontal cortex that is immediately ventral to the PL, provides cortical input involved in habit formation. It appears to have an opposite role in behavioral expression of habit than the PL does for actions: the IL is necessary for the performance of habitual actions (when inactivated after overtraining, outcome sensitivity is evident; Coutureau & Killcross, 2003) however it may not be required for the acquisition of S-R habits (Lingawi et al., 2016). Input from the IL to the DLS is thought to occur indirectly via its projections to the central nucleus of the amygdala (CeN). The CeN is thought to be involved in the robustness of responding to Pavlovian cues (Balleine & O’Doherty, 2010), which may explain its role in habitual responding to associated stimuli. When the CeN and DLS are functionally disconnected (though unilaterally intact and therefore still individually operative), habit formation is prevented, though this effect is specific to lesions of the anterior CeN and not the posterior CeN (Lingawi & Balleine, 2012). Importantly, the anterior CeN projects to the lateral substantia nigra pars compacta (SNC), which is responsible for a significant degree of the dopaminergic innervation of the DLS (Lingawi et al., 2016).

Role of Striatal Motor Pathways

Within the striatum there are two distinct populations of GABAergic medium spiny neurons (MSNs) that make up the direct and indirect motor pathways. Direct
pathway MSNs (dMSNs) selectively express excitatory D1 dopamine receptors, whereas indirect pathway MSNs (iMSNs) selectively express inhibitory D2 dopamine receptors (e.g. Smith, Bevan, Shink, & Bolam, 1998; Kreitzer & Malenka, 2008). The direct and indirect pathways differentially gate excitatory output from the thalamus to the sensorimotor cortex in movement initiation via their projections to the Globus Pallidus internal (See Box 1). Experimental manipulations in which the activity of one pathway is selectively enhanced demonstrate that increasing activity in the indirect pathway inhibits locomotion, whereas activating the direct pathway enhances locomotion (e.g. Cui et al., 2013; Graybiel, 1995; Megens et al., 2014, etc.). These results suggest that the two pathways have opposing effects on motor output. However, both pathways are concurrently activated during action selection (Cui et al., 2013), and recently a theory has emerged which holds that, while the direct pathway does facilitate the expression of a given voluntary movement, the indirect pathway does not counteract this expression but instead suppresses other, non-desired competing motor outputs (Cui et al., 2013; Dunovan & Verstynen, 2016). This theory holds, therefore, that the two pathways interact dynamically to fine-tune motor output. Critically, this cooperation requires that the relative strength of each pathway be carefully balanced, and subsequently that the timing with which the dMSNs and iMSNs synapse onto their targets in the globus pallidus internal be such that inhibition by the indirect pathway targets thalamic outputs that are correlated specifically with the undesired motor programs (O’Hare et al., 2016).
The presence of dopamine in the striatum is thought to facilitate movement via binding of D1 and D2 receptors in the direct and indirect pathway, respectively, resulting in enhanced direct pathway activation and suppressed indirect pathway activity. Striatal DA is therefore critical for movement initiation, as is evident in movement disorders such as Parkinson’s where striatal DA innervation is decreased (e.g. Kravitz & Kreitzer, 2012). These two pathways were canonically studied for their roles in generating movement,
however with emerging evidence for the importance of striatal dopamine in action
selection in the DMS and DLS (see above), have recently have become a target of interest
for their involvement in reinforcement learning and habit.

Optogenetic studies have provided evidence for the role of the direct and indirect
pathways in reinforcement. For example, Kravitz, Tye, and Kreitzer (2012) used a self-
stimulation optogenetic paradigm in which mice were trained to press a trigger that
would initiate laser illumination. Targeting neurons in the DMS, they found that mice
expressing ChR2 (an excitatory compound) in direct pathway neurons would increase
trigger-pressing over time, while mice expressing ChR2 in indirect pathway neurons
would decrease trigger-pressing. These findings suggest that activation of the direct
pathway in the DMS is reinforcing, while activation of the indirect pathway is punishing,
or reduces behavior. Additionally, self-stimulation of iMSNs vs dMSNs appears to
increase generalization of responding and decrease sensitivity to changes in response-
outcome contingencies (Vicente, Galvão-Ferreira, Tecuapetla, & Costa, 2016), which
relates to how habitual behavior is insensitive to changes in outcome value. Drew et al.
(2007) demonstrated that transgenic mice bred to reversibly overexpress D2 receptors in
the striatum showed lower operant responding on a progressive ratio schedule (a measure
of motivation) compared to controls, and this effect was attenuated when this
overexpression was reversed, suggesting that D2 binding may attenuate motivation for
reward.

1.3 Habit, Addiction, & the Effect of Estrogen

A hallmark of drug addiction is the progressive loss of control over drug-seeking
and consumption. One theory is that this progression is linked to the behavioral
transition from goal-directed A-O actions to automatic S-R habits: drug-taking begins as a motivated, goal-directed process but becomes compulsive and persists despite negative consequences (Ostlund & Balleine, 2008; Hogarth et al., 2013). As motor neurons throughout the striatum, including the DMS, DLS, are all modulated by dopamine, it is unsurprising that the processes in which they are involved are sensitive to being hijacked by substances that affect dopamine. Hence, drugs of abuse, which alter striatal dopaminergic function, may set the stage for maladaptive habit formation.

Habitual drug-seeking is well-documented in animal models (alcohol: Dickinson, Wood, & Smith, 2002; Corbit, Nie, & Janak, 2012; cocaine: Miles, Everitt, & Dickinson, 2003; Zapata et al., 2010; nicotine: Loughlin, Funk, Coen, & Lê, 2017; methamphetamine: Cox et al., 2016). A particularly elegant study conducted by Corbit, Nie, and Janak (2012) demonstrated habitual drug-seeking in rats trained to lever-press for alcohol. They lesioned the DLS and DMS at early and late time-points during training, and found that alcohol-seeking was only attenuated by DMS lesions when responding was still sensitive to devaluation, and only attenuated by DLS lesions when responding was insensitive to devaluation. Similarly, DLS inactivation was shown to restore sensitivity to devaluation in habitual cocaine seeking (Zapata et al., 2010). It has also been found that dopamine signaling increases in the DLS over the course of cocaine use (Willuhn, Burgeno, Everitt, & Phillips, 2012).

Amphetamines (AMPH) are known to increase striatal dopamine (Zetterström et al., 1983), and also to enhance habit formation in rats (e.g. Nordquist et al., 2007; Nelson & Killcross, 2006). In order to investigate the neural substrates of AMPH-enhanced progression to habitual behavior, Nelson and Killcross (2013) conducted a series of
studies in which they successfully demonstrated that the previously observed acceleration of habit formation in AMPH-treated rats was reversed by antagonism of D1 receptors but enhanced by antagonism of D2 receptors. This critical finding establishes a functional role of the direct and indirect pathway in habit formation when synaptic dopamine levels are high following amphetamine exposure. It has also been proposed that AMPH heightens the incentivizing properties of a reinforcer (Nordquist et al., 2007). It was also previously found that AMPH-sensitization increased spines on MSNs in the DLS and reduced spines on MSNs in the DMS (Jedynak et al., 2007), thereby increasing the responsiveness of the DLS to dopamine. Together, these findings suggest that higher degrees of striatal dopamine, due to exposure to AMPH, pushes striatal activity towards habit formation.

Estrogen & Addiction Behavior

Our laboratory is particularly interested in the study of estrogen and sex differences, and there is abundant evidence for sex differences in addiction behavior (for detailed review, see Becker & Hu, 2008), likely mediated by gonadal hormones. In humans, a marked sex difference in addiction behavior is telescoping, a phenomenon seen primarily in women which is characterized by an accelerated progression from initial drug use to dependence (Greenfield, Back, Lawson, & Brady, 2010; Haas & Peters, 2000). Additionally, preclinical data suggests that women tend to have a faster rate of consumption of drugs of abuse and have more difficulty quitting, especially during phases of the menstrual cycle when estrogen levels are
high or starting to decline. This is particularly related to the use of psychostimulants such as cocaine and methamphetamine in women (Becker & Hu, 2008). There are analogous findings in female rats as well: females more readily acquire cocaine self-administration than male rats (Lynch & Carroll, 1999).

The particular effects of gonadal hormones on addiction behavior can be more directly observed in animal studies in which ovariectomized (OVX) female or castrated (CAST) male animals can be compared to either intact animals, animals with hormone replacement, or before and after gonadectomy. When comparing responding for cocaine on a progressive ratio schedule (a measure of motivation; the number of responses required to earn a reinforcer increases over time) between OVX vs. OVX plus estradiol female rats, estradiol (E2) replacement led to greatly enhanced responding, suggesting that E2 augments motivation for cocaine (Becker & Hu, 2008). In fact, intact females tend to have higher breakpoints on progressive ratio schedules (when the animal stops responding) than males for cocaine and nicotine (Li et al., 2014), and these breakpoints are influenced by the estrous cycle (Roberts, Bennett, & Vickers, 1989).

Acquisition of cocaine self-administration is reduced in OVX animals, and restored to similar levels of intact females with estrogen replacement (Lynch, Roth, Mickelberg, & Carroll, 2001) and the same pattern is seen in response to amphetamines (Becker & Hu, 2008). This restored responding elicited by estrogen replacement in OVX animals is blocked by the estrogen receptor antagonist tamoxifen (Gillies & McArthur, 2010; Lynch et al., 2001). These effects are not seen, however, in males, whether intact, CAST, or treated with E2 or testosterone (Gillies & McArthur, 2010). Together, these behavioral data alone suggest that estrogen is a key factor in the sex differences observed...
in drug addiction behavior, particularly with psychostimulants such as cocaine and amphetamine.

_Estrogen in the Dorsal Striatum_

Findings from the past forty years have consistently demonstrated that estrogen, via multiple mechanisms such as availability, receptor density, and transporter affinity (Almey, Milner, & Brake, 2015) modulates striatal dopamine functioning. In an early study by Hruska & Silbergeld (1980), unilateral neurotoxic lesions were made in the striatum of male rats, and d-amphetamine was administered as a dopamine agonist. In males that were then treated with E2, there was an increase in dopamine receptors, and dopamine-dependent rotation behavior was rescued, providing seminal evidence for the influence of estrogen on striatal dopamine.

Treatment of OVX female rats with E2 has been shown to increase dopamine release (Shams et al., 2016) and turnover (Di Paolo, Rouillard, & Bedard, 1985) in the dorsal striatum. Additionally, the influence of estradiol treatment appears to be sexually dimorphic: amphetamine-induced dopamine release in the striatum is drastically attenuated by gonadectomy in females but not in males (Becker & Ramirez, 1981), and is restored only in OVX females following E2 treatment (Becker, 1990), a pattern which has also been observed with cocaine-induced dopamine release specifically in the DLS following treatment of gonadectomized males and females following estradiol benzoate treatment (Cummings, Jagannathan, Jackson, & Becker, 2014).

Estrogens also likely modulate the activity of the direct and indirect pathways via their effects on dopamine receptors. In cultured striatal neurons, treatment with E2 had
several effects: it blocked cellular response to D2 binding, but approximately doubled the
effect of D1 binding on enzymatic activity in neurons (Maus et al., 1989) and decreased
current through calcium channels in striatal MSN’s (Mermelstein, Becker, & Surmeier,
1996). Additionally, when estradiol benzoate was injected into both OVX and CAST rats,
there was a rapid (30-minutes following injection) decrease in dopamine binding at D2
receptors in striatum, but only in OVX rats (Bazzett & Becker, 1994). Additionally, both
acute (Lévesque & Di Paolo, 1988) and subchronic (Tonnaer, Leinders, & van Delft,
1989) administration of estradiol in OVX rats decreases the ratio of high to low affinity
striatal D2 receptors. In contrast, OVX leads to decreases in D1 receptor density
compared to intact females (Lévesque, Gagnon, & Di Paolo, 1989). Together, these data
suggest that the presence of estradiol in striatal motor pathways (in female rats only) may
enhance the functioning of the direct pathway, either by enhancing D1 activity, or
disinhibiting the direct pathway by blocking D2 binding or limiting GABAergic inputs
from the indirect pathway.

*Estrogen and Habit Formation*

Dopamine activity in the dorsal striatum and in the striatal motor pathways is
associated with habit formation, influenced by drugs of abuse, and affected by ovarian
hormones such as estrogen. However, the majority of research regarding the involvement
of these systems in habit formation has been done in male animals, or hasn’t compared
the response of males to that of females in a rigorous manner. One study looking at
devaluation by satiety of a sucrose reward following a Pavlovian cue-directed approach
behavior, did show that female rats appeared less sensitive to outcome devaluation than males (Hammerslag & Gulley, 2014). However, sex-differences in carbohydrate metabolism and sugar preference (Butera, 2010; Vieira-Marques et al., 2017; Mauvais-Jarvis, 2015) are problematic when using satiety to devalue rewards and to compare female and male animals, and didn’t behaviorally evaluate the transition from a goal-directed action to habit. In fact, a direct comparison of male and females in the development of habitual responding using reinforcer devaluation with LiCl has yet to be done.

The primary goal of the following studies was to determine if female and male rats show habitual behaviors at a similar level of reinforcer exposure. A secondary goal was to determine if methamphetamine (METH) enhances habit formation in ovariectomized females at the same level of reinforcement at which it does so in males, and if cycling estrogen interacts with METH to affect habit formation. A tertiary goal of these studies was to ascertain the degree of training that is sub-threshold to- and at the threshold of- habit formation in female rats, in order to be able to more precisely test factors that may attenuate or enhance habit formation in females.
CHAPTER 2. EXPERIMENT 1: SEX DIFFERENCES IN HABIT FORMATION IN RATS

2.1 Introduction

As mentioned previously, our laboratory is unaware of any prior studies in which the development of habitual S-R behavior in males and females has been compared by evaluating each sex with equivalent levels of training in the development of habitual S-R behavior using reward devaluation (RD) with LiCl. Thus, this was the primary aim of our first set of experiments. Previous research has identified that, using a lever-press operant response, both male (Dickinson et al., 1995) as well as female (Thrailkill & Bouton, 2015) rats are insensitive to outcome devaluation after being trained with 360 reinforcers (A-O contingencies) on an interval schedule, whereas 120 reinforcers maintains goal-directed behavior in males (Dickinson et al., 1995; Nelson & Killcross, 2006). Thus, we chose exposure to 240 reinforcers earned on a VI-30 schedule as an intermediate point for instrumental training for both sexes using a different operant response (nose-poking). We hypothesized, based on the literature reviewed previously in which estrogen appears to organize favorable conditions for habit formation, that females and males would differ in the degree of training required to render behavior insensitive to outcome devaluation. Running males and female within the same experiment is problematic because the presence of cycling females is perceived by males and thus, may alter male behavior. Moreover, males are larger, and grow in size significantly faster than females and hence may eat more pellets than females in all stages of the experiment. Finally, as iterated
above, there is a plethora of literature suggesting that DLS physiology significantly differs between males and females, with the male DLS being unaffected by gonadal hormones. Therefore, males and females were evaluated with exactly the same experimental methodology in separate experiments.

2.2 Methods

Animals

Adult Long Evans rats (Charles River, Quebec; 75-90 days old at the time of arrival), 26 female (Experiment 1A) and 21 male (Experiment 1B) were housed in same-sex pairs in a climate-controlled colony room maintained at 23°C with a 12-hr light-on-light-off cycle (7:00 A.M. to 7:00 P.M.). All testing occurred during the light phase of the cycle. All rats were given five days following arrival in the colony room to habituate before being gently handled in a consistent manner. Rats were on restricted feed to maintain a target weight of 85% of their ad libitum weights for the duration of the experiment. This was done by weighing rats daily and calculating the amount of food needed to maintain target weights. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

Apparatus

The training apparatus was comprised of six standard rat operant chambers (Med Associates, St. Albans, VT) kept within individual noise-attenuating cabinets ventilated by low-noise fans. In the center of the right-facing chamber wall was a head-entry port into which a hopper delivered a 45-mg sucrose pellet (Bio-Serv). To the right of the head entry was a nose-poke device (ENV-114, Med Associates) which emitted an infrared
beam; when animals performed a nose-poke, this beam was disrupted and signaled for the delivery of a sucrose pellet to the operant chamber based on the schedule of reinforcement being used. All data from the operant boxes was monitored and collected by MED-PC software (Med Associates).

Instrumental Training

At the beginning of each experiment, all rats were assigned to one of the six operant chambers, in which they were tested throughout the experiment. The house light in the chambers was illuminated at the beginning of each session, and would turn off automatically once the session was completed.

Magazine Training. All rats received two 30-minute sessions of magazine training, during which sucrose pellet reinforcers were presented non-contingently on a variable-time 60-s (VT 60-s) schedule.

Nose-poke acquisition. Following magazine training, all rats then received two sessions on a continuous schedule of reinforcement, in which every performance of a nose-poke was reinforced until the animal reached a total of 25 reinforcers in each session. Following these sessions were 6 sessions (2 per day for three days) of nose-poke acquisition training on a variable-interval 30-s (VI-30-s) schedule. These sessions terminated after rats had earned 40 reinforcers, for a total of 240 exposures over the course of training.

Reinforcer Devaluation (RD)

Following the final session of acquisition, half of the animals were randomly assigned to the paired group, with their cage-mates matched to the unpaired group. All
rats underwent a reinforcer devaluation paradigm which proceeded until criterion was met: all animals in the devalued (or paired) group had ceased all consumption of sucrose pellets. It was crucial to drive consumption to zero in the paired group during this procedure, to dissociate responding at test from any operant motivation for the reinforcer. During each session of RD, nose-poke responses were prevented by physical removal of the nose-poke holes from the operant chambers, and animals were freely delivered pellets on a VT 30-s schedule. On odd-numbered days in this paradigm, all rats were placed in the operant boxes, although only animals assigned to the paired group received sucrose pellets (starting with a total of 40 pellets on Day 1). Rats in the unpaired group were yoked in these sessions to a paired rat in the neighboring operant box; their sessions were terminated at the same time as their yoked counterparts’. Upon the completion of these sessions, all rats were immediately removed from the operant chambers and injected intraperitoneally (i.p.) with a 10 ml/kg dose of .15 M lithium chloride (LiCl) to induce nausea, then returned to their home cage.

On even-numbered days the same procedure was conducted, but on these days animals assigned to the unpaired group received sucrose reinforcers, while paired animals were placed in the operant chambers for the same duration as their yoked counterparts without receiving sucrose. Immediately following the termination of these sessions, all animals received an i.p. injection of 0.9% physiological saline of equivalent size to the LiCl injections. In this way, all rats experienced the same amount of time in the operant chamber during RD, and the same number of injections of both LiCl and saline; all that changed was access to the reinforcer, whereby only paired animals received illness on the days they received sucrose pellets. As devaluation continued, paired animals consumed
increasingly fewer pellets during their sessions, and the average number of pellets consumed would be presented the following day to the unpaired animals in their sessions.

*Testing Habit and Devaluation*

**Extinction Test.** On the day following the last day of RD, sensitivity to the devaluation was tested during a 12-minute session under extinction conditions: the nose-poke holes were unblocked, allowing for responses to be made, though no reinforcers were delivered during the session. Nose-pokes per minute were recorded by the computer software.

**Consumption Test.** On the day following the extinction test, devaluation of the sucrose reward was assessed during a brief (20 free presentations of the reinforcer on a VT-30s schedule) consumption test. During the consumption test, nose-pokes were again physically blocked.

**Reacquisition.** On the day following the consumption test, devaluation was assessed during a 30-minute reacquisition test, during which rats were placed in the operant boxes and were once again allowed to nose-poke for the reinforcer, which was delivered on a VI-30-s schedule. When re-exposed to the sucrose reinforcer, rats for which an aversion was successfully conditioned were expected to exhibit decreased nose-poking for the sucrose.

*Data Treatment*

Animals who failed to condition a taste aversion during RD (consumed all delivered pellets on all paired days throughout RD; 2 males, 2 females) and 1 male outlier (z=2.62; Field, 2007) were excluded from analysis.
2.3 Results

Experiment 1A.

Acquisition data were analyzed using a repeated-measures ANOVA with a within-subjects factor of training session (6) and a between-subjects factor of anticipated pairing groups (paired, \(n=12\); unpaired, \(n=14\)). Mean responses per minute across acquisition can be seen in Figure 1. Mauchly’s test indicated that the assumption of sphericity had been violated (\(\chi^2(14)=80.47, p<.001\)); therefore, degrees of freedom were corrected with Greenhouse-Geisser estimates of sphericity (\(\varepsilon=0.38\)). There was a significant main effect of session (\(F(1.91, 45.81)=28.21, p<.001\)) and no significant main effect of anticipated pairing group (\(F(1,24)<1, p=.57\)) or interaction between pairing group and session (\(F(1.91, 45.81)=.70, p=.50\)), indicating that females in both pairing groups successfully acquired nose-poking for sucrose pellets during training at an equivalent rate.
During RD, females in the paired group successfully acquired the conditioned taste aversion to the point where they rejected all delivered sucrose pellets (See Figure 2). As anticipated, unpaired animals consistently consumed all delivered reinforcers throughout RD.

Results from the extinction test indicated that there was no significant difference in mean nose-pokes-per-minute between paired ($M=8.19$, $SD=2.29$) and unpaired
(\(M=6.64, SD=4.38\)) females (\(t(15.99)=-1.11, p=.29\); see Figure 3), indicating a lack of sensitivity to outcome devaluation in these females.

![Extinction Test: Females](image)

**Figure 3.** Mean responses per minute during the extinction test for females.

The consumption test confirmed the success of the conditioned taste aversion in paired animals, in that all paired animals on average consumed zero of the delivered pellets at test whereas unpaired animals on average consumed all pellets. A 2 (pairing group; paired, unpaired) X 6 (time; five-minute bins) repeated measures ANOVA was conducted to evaluate differences in response rates between paired and unpaired animals during the reacquisition session (see Figure 4). Mauchly’s test revealed that the assumption of sphericity had been violated (\(\chi^2(14)=26.47, p=.02\)), therefore Greenhouse-Geisser corrections for degrees of freedom were used (\(e=0.68\)). A significant within-subject effect of time (\(F(3.4, 81.48)=5.19, p=.002\)) as well as a significant between-subjects effect of pairing group (\(F(1,24)=55.38, p<.001\)), were qualified by a significant time\(\times\)pairing group interaction (\(F(3.4, 81.48)=28.94, p<.001\)), indicating that all animals exhibited changing response rates across the reacquisition test, with paired and unpaired females significantly differing in response rate throughout. As can be seen in Figure 4,
unpaired animals show increased responding across the session, and paired animals inhibited their responding significantly across the test, confirming the success of the devaluation of sucrose in the paired group only.

![Figure 4. Mean responses per minute in reacquisition binned by five minute intervals.](image)

**Experiment 1B**

We ran a 6 (training session) by 2 (pairing groups; paired $n=9$, unpaired $n=12$) repeated measured ANOVA to examine acquisition for nose-poking for sucrose in males. Mauchly’s test revealed a violation of the assumption of sphericity ($\chi^2(14)=35.98$, $p=.001$), and Greenhouse-Geisser corrections for degrees of freedom were employed ($\varepsilon=0.61$). Results of this ANOVA showed that males significantly acquired nose-poking for sucrose pellets ($F(3.02, 57.46)=109.16, p<.001$; see Figure 5), and a lack of a significant main effect of anticipated pairing group ($F(1,19)=.51, p=.485$) or interaction
between pairing group and training session \((F(3.02,57.46)=.86, p=.466)\) indicate that paired and unpaired males acquired nose-poking for sucrose at an equivalent rate.

![Acquisition: Males](image)

**Figure 5.** Mean responses per minute across six acquisition sessions for males

Paired males successfully acquired the conditioned taste aversion, and ceased consuming all delivered sucrose pellets (see Figure 6).

![RD Consumption: Males](image)

**Figure 6.** Number of pellets consumed by paired males across sessions of RD
Results from the extinction test showed that paired males ($M=3.71$, $SD=1.23$) responded significantly less than their unpaired counterparts ($M=5.26$, $SD=1.43$; $t(19)=2.59$, $p=.02$; see Figure 7).

During the consumption test, unpaired males on average consumed all delivered pellets, whereas paired animals on average consumed zero. To examine the differences in responding between paired and unpaired males during reacquisition (see Figure 8), a 2 (pairing group; paired, unpaired) X 6 (time; five-minute bins) repeated measures ANOVA was conducted. A significant within-subject effect of time ($F(5, 95)=16.95$, $p<.001$), as well as a significant between-subjects effect of pairing ($F(1,19)=85.9$, $p<.001$) was qualified by a significant time x pairing group interaction ($F(5,95)=46.59$, $p<.001$). Like in Experiment 1A, this indicates that unpaired males significantly reacquired nose-poking for sucrose across the reacquisition test, whereas paired animals did not.
2.4 Discussion

At this level of instrumental training (240 reinforcers), females demonstrated habitual responding while males maintained goal-directed responding, confirming a sex difference in the development of habitual behavior as measured by sensitivity to reward devaluation. It should be acknowledged that previous research has demonstrated sexual dimorphisms in conditioned taste aversion, such that males require longer to extinguish a conditioned taste aversion than females (Chambers & Sengstake, 1976), and this effect appears to mediated by gonadal hormones (Chambers, 1985). However, this does not represent a confound to our findings, as habit is tested after the taste aversion is successfully conditioned (consumption is at zero) and prior to any presentations of the sucrose unpaired with illness (extinction of the conditioned taste aversion). Additionally, while it could be argued that females would more readily perform an action for a sucrose reward, as females appear to have a higher sweet preference than males (e.g., Valenstein, Cox, & Kakolewski, 1967), paired females did demonstrate that the reinforcing
motivational properties of the sucrose reinforcer were sufficiently devalued: like males, paired females reached criteria for a conditioned taste aversion during reward devaluation by reaching zero consumption of all delivered pellets. They also on average rejected all pellets during the consumption test and most importantly did not reacquire nose-poking for sucrose during reacquisition.
CHAPTER 3. EXPERIMENT 2: EFFECTS OF ESTRADIOL AND METHAMPHETAMINE ON HABIT FORMATION IN OVARIECTOMIZED RATS

3.1 Introduction

The previous experiment demonstrated that females exhibit habitual behaviors before males do when trained to an equivalent degree. As mentioned previously, estrogen, specifically estradiol, is associated with increased vulnerability to psychostimulants, and cycling ovarian hormones throughout the estrous cycle influence drug-seeking behavior and dopaminergic activity in dorsal striatum, which oversees habit formation. The aim of this experiment was to examine how cycling E2 and methamphetamine influence the progression from goal directed to habitual behavior. As mentioned previously, males trained to 120 reinforcers on an interval schedule responded habitually following pre-training exposure to amphetamine (Nelson & Killcross, 2006; 2013), whereas drug-free controls with the same training remained goal-directed. The goal of this experiment was to determine if the same were true in female animals, with and without cycling estrogens.

Because drug-seeking behavior is sensitive to fluctuations in hormones across the estrous cycle, we wanted to mimic rising and falling estradiol levels seen endogenously in intact, as opposed to chronically high or low estradiol. To isolate the effects of estradiol in this paradigm, we used OVX rats and replaced with estradiol only; progesterone can at times works in synergy with estrogen (e.g., response strategy selection, Korol & Pisani, 2015) but has also been observed to have opposing effects to estradiol (e.g. spatial memory, Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006).
In order to maintain estrogen receptor availability, we implanted all experimental animals with slow-releasing silastic capsules (see methods below) containing very low (approximately diestrus) amounts of estrogen.

For this experiment, following implantation of the silastic capsules, we divided our animals into a cycling estrogen group for which bolus injections of estrogen were given every four days to mimic the estrous cycle, and a low-estrogen control group which received sham bolus injections. Within each of those groups we then pre-treated half of the animals with methamphetamine (METH) with the other half receiving sham saline pre-treatment. While the experiments in males used dextro-amphetamine, it has been documented that the behavioral effects of dextro-amphetamine and methamphetamine do not differ (for discussion, see da-Rosa et al., 2012), therefore we chose to use methamphetamine for its clinical significance as an illegal drug of abuse. We hypothesized that either METH alone or METH and cyclic estradiol would enhance habit formation in OVX rats.

3.2 Methods

Animals

Animals for this experiment were 68 naïve, previously ovariectomized (OVX) female Long-Evans rats (Charles River, Quebec), 75-90 days old at the time of arrival. Rats were housed in pairs in a climate-controlled colony room maintained at 23 °C with a 12-hr light-on-light-off cycle (7:00 A.M. to 7:00 P.M.). All testing occurred during the light phase of the cycle. All rats were given five days following arrival in the colony room to habituate before being gently handled in a consistent manner. Rats were
maintained on a restricted diet in order to maintain a target weight of 85% of their *ad libitum* weights for the duration of the experiment. This was done by weighing rats daily and calculating the amount of food needed to maintain target weights. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

**Hormone delivery**

Continuous low-levels of E2 were delivered to all animals via 10 mm silastic capsules (0.058” ID, 0.077” OD, Fisher Scientific) implanted subcutaneously (s.c.) under isoflurane anesthetic at the scruff of the neck between the scapulae. Capsules contained 5% E2 (Tocris) and 95% cholesterol (Sigma). These capsules have been shown to steadily release approximately diestrus levels of E2, ~20 pg/ml (e.g. Almey et al., 2013). After five days of recovery from surgery, half of the animals were randomly assigned to a cycling estrogen group; to mimic estrus cycle, once every 4 days rats in the cycling estrogen group were given bolus s.c. injections of 10 µg/kg body weight (bw) doses of E2 dissolved in canola oil, to simulate proestrus levels of E2. Control groups received equivalently-sized s.c. injections of oil only. Bolus injections were given in a staggered manner, in order to mimic natural variability in estrous phase seen within a naturally cycling population of female rats.

**Drug Pretreatment**

After one week of bolus injections (so that all animals had received at least one 4-day “cycle”) rats received 8 consecutive daily intraperitoneal (i.p.) injections of 2.5 mg/kg body weight of Methamphetamine hydrochloride (Penro Specialty Compounds,
Colchester, VT) (methamphetamine group) or an equivalently sized injection of 0.9% physiological saline (vehicle group) followed by 3 days of washout to allow for methamphetamine elimination. All rats then received a low methamphetamine dose (0.3 mg/kg) to test for methamphetamine-induced sensitization of locomotor activity as a verification of drug exposure.

**Apparatus**

The same apparatus was used for this experiment as was used in Experiment 1.

**Instrumental Training**

At the beginning of each experiment, all rats were assigned to one of the six operant chambers, in which they were tested throughout the experiment. The house light in the chambers was illuminated at the beginning of each session, and would turn off automatically once the session was completed.

**Magazine Training.** Magazine training was the same as in Experiment 1.

**Nose-poke acquisition.** Following magazine training, all rats then received two sessions on a continuous schedule of reinforcement, in which every performance of a nose-poke was reinforced until the animal reached a total of 25 reinforcers in each session. Following these sessions were three daily sessions of nose-poke acquisition training on a variable-interval 30-s (VI-30-s) schedule. These sessions terminated after rats had earned 40 reinforcers, for a total of 120 exposures over the course of training.
Reinforcer Devaluation

The same RD procedure was used for this experiment as the one used in Experiment 1.

Testing Habit and Devaluation

The same extinction test, consumption test, and reacquisition test were used in this experiment as those described in Experiment 1.

3.3 Results

All animals reached criterion during acquisition and RD, therefore all except one outlier ($z=2.05$) were included for analysis.

Acquisition

To examine acquisition in all four groups, a repeated measures ANOVA was conducted with a within-subjects factor of training session (3 sessions), and between subject factors of hormone group (2; control, estrogen), drug-pretreatment (2; meth, vehicle) and anticipated pairing groups (2; paired, unpaired. A violation of the assumption of sphericity was detected with Mauchly’s test, ($\chi^2(2)=11.45, p=.003$), and Huynh-Feldt corrections of degrees of freedom were used ($\epsilon=0.85$). A significant main within-subjects effect of training session indicated that all groups significantly acquired nose-poking for sucrose ($F(1.95,116.92)=123.51, p<.001$; see Figure 9). There was no significant main effect of pairing group ($F(1.60)=1.80, p=.185$) or interaction between pairing group and session ($F(1.95, 116.92)=0.45, p=.634$), indicating that anticipated paired/unpaired animals in all groups acquired nose-poking for sucrose at an equivalent
rate. While the main effect of hormone group was only marginally significant \((F(1,60)=3.18, p=.080)\), there was a significant training session \(x\) hormone group interaction \((F(1.95, 116.92)=6.28, p=.003)\), such that on the final day of acquisition, collapsed across all other groups, animals in the estrogen group exhibited a higher average response rate \((M=15.23, SD=5.22)\) than animals in the control group \((M=12.59, SD=3.92)\).

![Figure 9](image.png)

**Figure 9.** Mean responses per minute during acquisition, separated by hormone replacement and drug pretreatment groups. All groups significantly acquired nose-poking for sucrose.

Additionally, all paired animals in each group successfully acquired the taste aversion for sucrose, reaching criteria of zero pellets consumed by the end of RD (See Figure 10). On even-numbered days, unpaired animals consumed all delivered pellets for the duration of RD.
Extinction Test

A 2 (Hormone Replacement: control or estrogen) x 2 (Drug pre-treatment: vehicle or meth) x 2 (Pairing: paired or unpaired) factorial ANOVA revealed a significant main effect of pairing ($F(1,60)=37.9, p<.001$). There was no significant hormone replacement x drug pre-treatment x pairing interaction ($F(1,60)=1.765, p=.189$). Further, pairwise comparisons using estimated marginal means and Least Significant Difference adjustment for multiple comparisons revealed that within each group, paired animals responded significantly lower than unpaired animals (all $ps<.05$) indicating that within all groups, animals were still goal-directed (see Figure 11).
Figure 11. Mean responses per minute during the extinction test. Within each group, paired animals responded significantly less ($p<.05$) than unpaired animals.

**Consumption Test and Reacquisition**

All animals behaved as expected during the consumption test: paired animals on average consumed zero pellets delivered whereas unpaired animals on average consumed all pellets. Successful devaluation of the sucrose reinforcer was evaluated with a repeated measures ANOVA in six 5-minute bins. Mauchly’s test indicated a violation of the assumption of sphericity ($\chi^2(14)=126.83$, $p<.001$) and Greenhouse-Geisser corrections for degrees of freedom were used ($\varepsilon=0.472$). There was a significant main effect of time ($F(2.36, 141.64)=11.95$, $p<.001$) and pairing group ($F(1,60)=138.64$, $p<.001$) and a significant 2 (pairing) x 6 (bin) interaction ($F(2.36,141.64)=33.12$, $p<.001$), indicating that, collapsed across groups (hormone replacement and drug pre-treatment), paired and unpaired animals responded significantly differently from one another. Paired animals
significantly increased responding across the test and unpaired animals significantly decreased responding (see Figure 12).

![Figure 12](image.png)

**Figure 12.** Mean responses per minute binned by 5-minute intervals. There was a significant difference between paired and unpaired animals, collapsed across hormone replacement and drug pretreatment group, such that unpaired animals reacquired nose-poking for sucrose.

This, along with the results of the consumption test, confirms that the differences observed during the extinction test were the result of a successfully conditioned aversion to sucrose in the paired animals, and that animals in the paired group retained the memory of that aversion.

### 3.4 Discussion

At this low sub-threshold level of training, methamphetamine, with or without cycling E2 replacement, was not sufficient to produce accelerated habit formation in female rats: all paired groups remained sensitive to the devaluation of sucrose. Interestingly, this result differs from previously published results for male rats, in which
methamphetamine pre-exposure at this same level of training produced habitual behavior (Nelson & Killcross, 2006; 2013). However, it should be noted that there were several differences in methodologies that would make us wary to identify this discrepancy with the literature as a sex difference in the role of amphetamines in habit formation: our experiment used a different operant (nose-poking vs lever-pressing), a more rigorous, and lengthier devaluation procedure, and a different strain of rats than the aforementioned studies using male animals.

There are two distinct questions that arise from these results. It is possible that the influence of methamphetamine on habit formation depends on a different hormonal milieu than that tested in our experiment. It would be beneficial to run a future study in which a group was included for replacement with both estrogen and progesterone in order to rule out this possibility.

Another important possibility that may explain the results of this experiment is that we did not train our females to a degree where they were sub-threshold to habit. The previously mentioned studies done with males and amphetamine rely on this sub-threshold level of training, and it’s possible that 120 reinforcers, using this operant procedure and our devaluation paradigm, may not be enough training whereby methamphetamine could facilitate the expression of habitual responding. In order to accurately assess the role of methamphetamine or estradiol/OVX, it would be crucial to identify the level of training that is subthreshold to habit in female rats.
CHAPTER 4. EXPERIMENT 3: SUB-THRESHOLD DEGREE OF TRAINING FOR HABIT FORMATION IN FEMALE RATS

4.1 Introduction

Experiment 3 aimed to address the possibility that the 120 reinforcers earned during acquisition in Experiment 2 was not truly subthreshold training to habit formation for female rats using the nose-poke operant. If the effect of methamphetamine was being masked in Experiment 2 by setting the training threshold too low, we may yet see that habit formation is enhanced by methamphetamine in intact females as it was in males (e.g. Nelson & Killcross, 2006). As mentioned previously, subthreshold training in males appears to be around 120 reinforcers on an interval schedule (Dickinson et al., 1995; Nelson & Killcross, 2006); while our data from Experiment 1 suggests that females form habits earlier than males, indicating that subthreshold training for females should be lower for females as well, it should be noted that we are using a different operant response, nose-pokes, whereas the aforementioned studies in males used lever-pressing. The aim of this experiment was to systematically assess increasingly lower levels of training in order to identify subthreshold training parameters for intact females on a nose-poke operant. We incrementally decreased training in our groups by 40 reinforcers, or one acquisition session’s worth of training in the prior experiments. Knowing that females were in habit at 240 reinforcers, we trained a Long training group to 200 reinforcers, and a Short training group to 160 reinforcers. We hypothesized that the Long group would be in habit, and that our Short group would be in action, indicating a range of subthreshold training between 160-200 reinforcers.
4.2 Methods

Animals

Animals for this experiment were 23 naïve, female Long-Evans rats (Charles River, Quebec), 75-90 days old at the time of arrival. Rats were housed in pairs in a climate-controlled colony room maintained at 23 °C with a 12-hr light-on-light-off cycle (7:00 A.M. to 7:00 P.M.). All testing occurred during the light phase of the cycle. All rats were given five days following arrival in the colony room to habituate before being gently handled in a consistent manner. Rats were on restricted feed in order to maintain a target weight of 85% of their ad libitum weights for the duration of the experiment. This was done by weighing rats daily and calculating the amount of food needed to maintain target weights. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

Apparatus

The same apparatus was used for this experiment as in Experiment 1.

Instrumental Training

Animals were randomly assigned (with matched cage-mates) to one of two training groups: the long group (200 reinforcers earned during acquisition) and the short group (160 reinforcer earned during acquisition). The beginning of training for each group was staggered by one day, with the Long group beginning earlier. This way, acquisition ended on the same day for both groups, thereby controlling for retention interval differences between the last day of training and the extinction test. Within each training group, rats were randomly assigned to one of six operant chambers in which they
were tested throughout the experiment. The house light in the chambers was illuminated at the beginning of each session, and would turn off automatically once the session was completed.

**Magazine Training.** Magazine training was the same as in Experiment 1.

**Nose-poke acquisition.** Following magazine training, all rats then received two sessions on a continuous schedule of reinforcement, in which every performance of a nose-poke was reinforced until the animal reached a total of 25 reinforcers in each session. Following these sessions were 4 (short group) or five (long group) daily sessions of nose-poke acquisition training on a variable-interval 30-s (VI-30-s) schedule. Each of these sessions terminated after rats had earned 40 reinforcers, for a total of 160 and 200 reinforcer exposures in the short and long groups, respectively.

**Reinforcer Devaluation**

The same RD procedure was used for this experiment as the one used in Experiment 1.

**Testing Habit and Devaluation**

The same extinction test, consumption test, and reacquisition test were used in this experiment as those described in Experiment 1.

**4.3 Results**

One animal failed to acquire the conditioned taste aversion during RD (consumed all delivered pellets on all paired days throughout RD; short group) and was therefore excluded from analysis.
Acquisition

All animals significantly acquired nose-poking for sucrose pellets, as confirmed by repeated measures ANOVA, $F(3, 57)=77.82, p<.001$. Comparing the first four sessions of acquisition, the long and short group acquired at an equivalent rate (non-significant main effect of training group, $F(1, 19)=1.40, p=.251$; See Figure 13) as did anticipated paired and unpaired groups across both training groups (non-significant main effect of pairing group, $F(1, 19)=1.32, p=.264$).

![Figure 13. Mean responses per minute across acquisition sessions, by training group. All animals significantly acquired nose-poking at equivalent rates.](image)

Additionally, all paired animals successfully acquired the taste aversion for, reaching criteria of zero pellets consumed by the end of RD (See Figure 14). On even-numbered days, unpaired animals consumed all delivered pellets for the duration of RD.
Figure 14. Number of pellets consumed by paired animals in both training groups across sessions of RD. Taste aversion for sucrose was acquired at equivalent rates in both training groups.

**Extinction Test**

A 2 (Training Group) x 2 (Pairing) factorial ANOVA revealed a lack of significant main effects of training group or pairing ($p=.20; p=.81$, respectively) and no significant training X pairing interaction ($F(1,20)=0.15, p=.70$). Pairwise comparisons using estimated marginal means and Least Significant Difference adjustment for multiple comparisons further revealed that there was no significant difference between response rate (mean nose-pokes per minute) between Long group paired ($M=6.10, SD=2.99$) and unpaired ($M=5.94, SD=2.46$) animals ($p=.66$), or between the Short group paired ($M=4.33, SD=2.36$) and unpaired ($M=4.99, SD=2.30$) animals ($p=.92$; See Figure 15).
Consumption Test and Reacquisition

All animals behaved as expected during the consumption test: paired animals on average consumed zero pellets whereas unpaired animals on average consumed all pellets. A 6 (time: 5-minute bins) by 2 (training group: long or short) by 2 (pairing: paired or unpaired) repeated measures ANOVA was conducted to examine group differences in reacquisition. Mauchly’s test revealed a violation of the assumption of sphericity ($\chi^2(14)=84.54, p<.001$), therefore Greenhouse-Geisser corrections for degrees of freedom were employed ($\varepsilon=0.32$). There were significant main effects of time ($F(1.59, 30.27)=8.31, p=.003$) and of pairing group $F(1,19)=84.49, p<.001$ and a significant bin x pairing group interaction ($F(1.59, 30.27)=17.86, p<.001$), indicating that across time during the reacquisition test and collapsed across training group, paired and unpaired animals responded significantly differently from one another. Further, there was not a significant main effect of training group ($F(1,19)=84.49, p=.424$), nor a significant time x
training group x pairing group interaction \((F(1.59, 30.27)=1.09, p=.337)\) indicating that paired and unpaired animals in both training groups reacquired equivalently. Figure 16 illustrates that paired animals significantly increasing responding across the test and unpaired animals significantly decreasing responding, failing to reacquire nose-poking for sucrose.

![Reacquisition Graph](image)

Figure 16. Mean responses per minute in reacquisition binned by 5-minute intervals.

4.4 Discussion

Animals in both the Long and Short training groups were insensitive to the devaluation of sucrose, suggesting that both levels of training were sufficient to produce habitual behavior in intact female rats. While this study aimed to find subthreshold training to habit in intact females, we were not successful, and future studies using less training will be required to identify the “tipping point” of training at which most intact females will be goal-directed.
CHAPTER 5. GENERAL DISCUSSION

There are several important findings from this series of experiments. First, there is a sex difference in the degree of training required to render responding for sucrose insensitive to reward devaluation with LiCl, such that females demonstrate habit at a level of training where males are still behaving in a goal-directed manner. Secondly, while the evidence from the literature indicates female vulnerability to the effects of psychostimulants, there was no effect found for ovariectomy, hormone replacement, or pre-treatment with methamphetamine on enhancing habit formation, in that all groups from Experiment 2 exhibit goal-directed responding for sucrose at test. While Experiment 3 sought to identify a degree of training in intact female rats that was just sub-threshold to habit, groups trained with 200 reinforcers and 160 reinforcers earned during acquisition on a variable interval schedule both demonstrated insensitivity to reward devaluation, suggesting that the degree of training that is subthreshold to habit formation using the nose-poke operant in intact females is lower than either degree of training used in Experiment 3.

The sex difference in habit formation found in Experiment 1 is particularly interesting in light of the literature regarding estrogen, addiction, and learning. Work by Korol and colleagues (for review, see Korol, 2004) suggests that estrogen state influences selection of a learning strategy: high estrogen states bias strategy selection away from striatal-dependent strategies towards those that are hippocampally-mediated. Additionally, E2 replacement in OVX animals drastically impairs DLS-dependent Set 1 learning in comparison to non-replaced animals in an extradimensional set-shifting paradigm (Lipatova et al., 2016). Further, selective agonism of both the ER-alpha and
ER-beta estrogen receptors impairs DLS-dependent response learning (Pisani, Neese, Katzenellenbogen, Schantz, & Korol, 2016). These behavioral findings suggest that high estrogen levels impair DLS-dependent learning, which at first consideration appears to directly contrast with our results from Experiment 1 where DLS-dependent habit formation is seen in intact female rats at an earlier point in learning (training) than male rats. As previously reviewed, high estrogen states also appear to impact the dorsal striatum on a neurological level. Firstly: the increase in DA release onto the dorsal striatum seen following the application of estrogen (e.g. Di Paolo et al., 1985) should have a net effect of enhancing the strength of the direct pathway by binding at excitatory D1 receptors, the activity of which is associated with the expression of habitual motor behaviors (e.g., Nelson & Killcross, 2013). Additionally, estrogen downregulates high affinity inhibitory D2 receptors in the indirect pathway over time (Lévesque, Gagnon, & Di Paolo, 1989), which indicates that high estrogen states over time leads to decreased sensitivity of the indirect pathway to modulation by dopamine.

We believe that this evidence from the literature as well as the recent model of how the direct and indirect pathway function cooperatively to produce motor output provide a theoretical framework for interpreting our results from Experiment 1. The majority of the studies reviewed previously examined the isolated effects of estrogen by replacing estrogen in OVX or CAST animals at acute or chronic high levels. We hypothesize that this high estrogen state changes the relative activation of both the direct and indirect pathway in the DLS, and thereby the afferent timing of their inputs to the globus pallidus internal. We suspect that the disruption of this carefully-timed process dysregulates the proper functioning of the DLS to most efficiently associate stimuli with
motor responses, and can explain the impairments observed in DLS-dependent learning in OVX females replaced with high, proestrus levels of estrogen (e.g., Korol, 2004). In Experiment 1, intact females were trained and tested across all phases of the estrous cycle; we postulate that the presence of the complete gamut of cycling gonadal steroids in females—estrogen as well as progesterone and testosterone—serves to maintain the level of relative activation of both pathways that is most optimal for efficiently pairing stimuli and motor outputs, and this may lead to enhanced habit formation in females. It would follow, then, that intact males, who lack cycling sex hormones, may exhibit a slower development of automatized stimulus-response behavior as a result of a less-efficient or finely-tuned striatal motor system. We are currently testing this hypothesis by enhancing direct and indirect pathway activation with pathway-specific viral vectors in female and male rats.

Given the theoretical framework established by our results in Experiment 1, one of the aims of Experiment 2 was to parse out if cycling estrogen alone (e.g., in lieu of constant high levels of estrogen used previously in the literature) could account for enhanced habit formation in females, or if the whole gamut of cycling ovarian hormones is needed. In addition to the potential role of cycling sex hormones in habit formation, there is evidence that fluctuating sex hormones affect other addiction-related behaviors, especially in regards to psychostimulants. For example, data suggests that women experience the subjective effects of psychostimulants differently at different stages of the menstrual cycle (Becker & Hu, 2008). However, the majority of research in animals looking at sex differences in psychostimulant-related behavior (e.g. conditioned place preference, acquisition of self-administration of cocaine or amphetamine, break-points on
progressive-ratio schedules), as well as the role of estrogen in female vulnerability to psychostimulants at a neural level (e.g., amphetamine- and cocaine-induced dopamine release in the striatum) have been done using static (not cycling) hormone replacement. By using cyclical E2 replacement in Experiment 2, we aimed to shed light on the nature of the interaction between cycling estrogen and psychostimulants.

Unfortunately, the results of Experiment 2 are limited in their interpretability: while there was no effect found for ovariectomy, hormone replacement, or pre-treatment with methamphetamine on enhancing or attenuating habit formation, the degree of training in this experiment may have masked some of these effects. While using the degree of training that prior research has identified as sub-threshold to habit in males is a reasonable starting-point, it is not a valid assumption that sub-threshold for females performing a different operant response (nose-pokes as opposed to lever-pressing) would be the same. We are therefore unable to confidently attribute the lack of habit in our groups to the cyclical hormone delivery we employed, or the lack of cycling progesterone and testosterone, and do not view these results as definitive evidence for a lack of a methamphetamine effect on habit formation in females. In a study conducted by Nelson and Killcross (2006), male rats were pre-exposed to low doses of amphetamine prior to undergoing instrumental lever-press training and reward devaluation in a paradigm similar to those used in Experiment 2. At test, control animals were still in action, however amphetamine-exposed male rats were responding habitually, as indicated by an insensitivity to devaluation of the outcome. As reviewed previously, this amphetamine-induced acceleration of habit formation in males is reversed by D1 receptor antagonism, and enhanced by D2 antagonism (Nelson & Killcross, 2013); since estrogen enhances D1
activity and attenuates D2 activity, as cited above, this suggests that estrogen may work cooperatively with amphetamines to enhance habit formation in females. Moreover, the literature strongly indicates that females display a particular vulnerability to the effects of psychostimulants, and that estrogen plays a modulatory role. For example, conditioned place preference for cocaine, which is seen in both male and female rats, is attenuated by OVX in females, but is potentiated E2 or E2 and progesterone replacement (Russo et al., 2003). In line with this finding, conditioned place preference for amphetamine is only seen in OVX animals if they’ve had replacement of E2 or E2 and progesterone (Silverman & Koenig, 2007). In light of this literature taken together, we remain unconvinced that our results from Experiment 2 rule out the possibility that methamphetamine could accelerate habit formation in females.

It is important to acknowledge that the “vulnerability” studies mentioned above were among those that used the standard static hormone-replacement strategy, and it could be argued that that psychostimulant vulnerability in females, like impairment in DLS-dependent behaviors, is related to high estrogen states specifically. However, as mentioned previously, this static replacement of estrogen and/or estrogen with progesterone in CAST males does not have the same enhancing effects on drug-related behaviors as it does in OVX females (Gillies & McArthur, 2010), indicating that there is something about being female, other than estrogen state or hormonal milieu, that facilitates female vulnerability to psychostimulants, and may underlie the response of the female system to amphetamines. One of the nuances in the study of sex differences comes from identifying whether or not the mechanism for a given effect is organizational or activational in nature. Briefly, the seminal organizational-activational hypothesis
(Phoenix, Goy, Gerall, & Young, 1959) held that gonadal hormones “organized” or established male and female tissue, including the brain, during development, and that these tissues become activated by gonadal hormones that come online during puberty. When hormone replacement in CAST males doesn’t elicit the same behavioral or physiological outcome as it does in females, this may reflect an organization of the female brain that differs from that of the male brain, which allows for activation by circulating hormones in females only. For example, only in OVX females, and not CAST males, does E2 enhance acquisition of cocaine self-administration (Jackson, Robinson, & Becker, 2006) and amphetamine-induced dopamine release in striatal tissue (Becker, 1990), suggesting that there are structural, organizational properties of being female that allow for these effects to be seen following activation by gonadal hormones. The canonical organizational-activational hypothesis has been modified in recent years to accommodate emerging evidence of sex differences that are associated with genes found on sex chromosomes (for review, see Arnold, 2009). Of particular interest for our work, possessing two X sex chromosomes (despite gonadal phenotype) has been associated with faster instrumental habit formation in transgenic mice (Quinn, Hitchcott, Umeda, Arnold, & Taylor, 2007). In light of this, it is necessary to consider our results from Experiment 2 with the understanding that sex differences in habit formation may not be mediated by organizational-activational mechanisms, but rather by genetic ones.

Additionally, as mentioned previously, it may be that cycling progesterone and testosterone along with estrogen are necessary to facilitate an amphetamine effect on habit formation in females. As addressed previously, many psychostimulant effects in OVX females are seen without progesterone replacement. In fact, progesterone can even
have an opposing effect in these interactions: unlike when estrogen was administered to OVX rats (without progesterone), progesterone administered alone (without estrogen) to OVX rats was not found to effect DA-enhanced amphetamine release or amphetamine-related behavior (Dalla & Shors, 2009; van Haaren, van Hest, & Heinsbroek, 1990). Additionally, progesterone alone in OVX animals inhibited conditioned place preference for cocaine, which is the opposite effect from what is seen when OVX animals are replaced with estrogen alone or estrogen in combination with progesterone (Russo et al., 2003). It is possible therefore that progesterone is not necessary in the system to observe enhanced habit in females. However, we emphasize that the relative activity of the indirect and direct pathways is in some way modulated by changes in several ovarian hormones—including progesterone and testosterone—over the estrus cycle that may be important in stimulus-response learning and the development of habitual behavior.

In order to further investigate the role of sex—be it organizational or activational—or methamphetamine in habit formation, it first will be necessary to identify the degree of training that is sub-threshold to habit formation in intact female rats. Having this information will allow us to test different manipulations—cycling vs static, CAST vs OVX, replacement with just E2 or with the whole gamut of gonadal steroids—can attenuate or enhance habit formation. Experiment 3 sought to identify this sub-threshold degree of training, however groups trained with 200 reinforcers and 160 reinforcers earned during acquisition on a variable interval schedule both demonstrated insensitivity to reward devaluation, suggesting that the degree of training that is subthreshold to habit formation using the nose-poke operant in intact females is lower than either degree of training used here. Our laboratory has recently generated
preliminary data in intact female rats in which the degree of training was decreased to 140 reinforcers earned on a variable interval schedule. Based on the findings from Experiment 2, in which OVX females with cyclic estradiol replacement were goal-directed after 120 reinforcers on the same schedule, and the findings from Experiment 3 that intact females were in habit at 160 reinforcers, this degree of training may be the tipping point. As of now, our data are inconclusive, although it appears as though there is no significant difference between our paired and unpaired females, indicating habit at this level of training as well. It is possible that we are narrowly missing the range of training that is required (130 reinforcers), or that intact females behave differently than OVX females with E2 replacement, such that 120 reinforcers or lower is sub-threshold for intact females. However, there are some methodological changes we could employ before moving on to shorter training.

Our extinction test is fairly brief: we use a 12-minute extinction test, based on what was used in the literature on males with amphetamine pretreatment (Nelson & Killcross, 2006) as well as in other studies of habit formation (e.g. Corbit et al., 2012). However, it is not uncommon to use a 20-minute (Adams & Dickinson, 1981; Adams, 1982) or 30-minute (e.g. Dickinson et al., 1995; Clemens, Castino, Cornish, Goodchild, & Holmes, 2014) test under extinction conditions. It is possible that our test of habit is too brief to observe a separation between paired and unpaired animals in our groups, especially if animals are within the threshold range of habit, which may drive mean responding rates higher for longer periods of time and thereby mask the detection of an action. However, response rates are overall very low in both the paired and unpaired groups by the end of our 12-minute test (nearly zero; as expected under extinction
conditions). Due to these floor effects in both groups, a longer extinction test, therefore, likely would not reveal group differences. This provides us with confidence that we have accurately tested habitual responding in the 12-minute test.

Our laboratory is still testing different degrees of training for the sub-threshold point in female rats, and intend to re-test the influence of methamphetamine and hormone milieu on habit formation using this amount of training. We also plan to assess the organizational vs activational properties of our target effects by running identical studies in CAST male rats. Finally, we also plan to investigate factors which may be protective against habit formation, or facilitate the breaking of an established habit. Our current findings indicate that females form habitual behaviors earlier than males, and the literature suggests a similar pattern of enhanced progression from the onset of drug use to dependence in human women. Ultimately, we hope to elucidate the connection between habit formation, addiction, and psychostimulant vulnerability in females in order to contribute to more targeted interventions for women suffering from addiction.
CITATIONS


