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Determining The Effects Of Castration And Estrogen Replacement On Habit Development In Adult Male Long Evans Rats

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DETERMINING THE EFFECTS OF CASTRATION AND ESTROGEN REPLACEMENT ON HABIT DEVELOPMENT IN ADULT MALE LONG EVANS RATS

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ABSTRACT

During instrumental learning, behavior transitions from goal-directed actions to habits. Goal-directed behavior is driven by the value of the outcome while habitual responding develops with increased training and is characterized by the automaticity with which it occurs independent of outcome value. The dorsal striatum, one of the many regions of the brain that has been implicated in the conversion of goal-directed actions to habits, is sexually dimorphic, and ovarian hormones are known to modulate the expression of habit in females. The present experiments were performed to begin deciphering how gonadal steroid hormones influence habit development in male rats. In each experiment, animals were trained to nose-poke for sucrose pellet reinforcers on a variable-interval (VI) schedule of reinforcement. In ½ of the males, the sucrose was then devalued through a conditioned taste aversion which was achieved by pairing the sucrose pellet with injections of lithium chloride (LiCl) to induce nausea. Classification of responding in each experiment occurred when animals were tested under an extinction condition. Devalued animals who remained goal-directed were expected to decrease responding for the devalued sucrose. Conversely, animals in habit, and therefore, insensitive to devaluation, were expected to respond at a similar rate as their non-devalued counterparts.

Experiment 1 examined the role played by gonadal steroid hormones in the development of habit in male rats by removing circulating steroid hormones through castration. When trained to 160, 240, 320, and 400 reinforced exposures, castrated (CAST) male rats express habitual behavior with training to 240 reinforced exposures which is much earlier in instrumental learning than habitual responding is observed in intact males. These results indicate that male sex hormones play a role in delaying habit development in intact males. Results from Experiment 1 and the literature on high estrogenic states on behavior in female rodents motivated us to conduct Experiment 2 to begin studying the effects of estradiol replacement on habit development in CAST males. Following the same paradigm as was employed in Experiment 1 with training to 160 reinforcers, goal-directed behavior was observed in estradiol (E2)-replaced CAST males. These results alone cannot be used to conclude how E2 specifically is acting on behavior in intact males since this was a small amount of training and future research on responding following additional training would need to be analyzed to ascertain exactly how these hormones control behavior. Estrogens have been found to have opposite effects on behavior between males and females and may play a role in the sex difference observed in habit formation in intact male versus female rodents, however, intact males are also constantly exposed to androgens in addition to estrogens. Thus, studies using the same methodology on the effects of androgens on habit development are planned.

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CHAPTER 1: LITERATURE REVIEW

1.1. Defining Actions and Habits

Instrumental learning occurs when behaviors are strengthened or weakened by their consequences resulting in their continuation or cessation, respectively. There are two forms of instrumental learning: goal-directed actions and habits. Early on in instrumental learning, behaviors are goal-directed or action-outcome (A-O) associations. As the name A-O association implies, these behaviors are controlled by the relationship between an action and the rewarding value of its outcome. Because goal-directed behaviors are influenced by the value of their outcome, they are consequently sensitive to changes in the value of the outcome (Adams & Dickinson, 1981).

With enough training and exposure of an action paired with an outcome in a specific learning environment, goal-directed behavior can transition to stimulus-response (S-R) associations (Dickinson, 1985). When S-R associations become dominant, the rewarding value of the outcome no longer drives the behavior; instead, the contextual stimuli of the learning environment cause the behavior reflexively and without conscious processing (Adams, 1982; Dickinson, 1985). These behaviors that begin to occur automatically become classified as habits.

Although habitual behaviors dominate over goal-directed actions and may be adaptive by decreasing the cognitive resources needed to perform a task (Lingawi, Dezfouli, & Balleine, 2016), they may also be maladaptive if harmful behaviors such as compulsive behaviors or drug-seeking become habitual. Extensive research has been conducted on how psychopathologies such as post-traumatic stress disorder, obsessive-

compulsive disorder, and addiction to drugs-of-abuse have origins in a dysregulation of goal-directed actions and habits (Goodman, Leong, & Packard, 2012; Belin et al., 2013; Ferreira et al., 2017). It is important to note that the transition from goal-directed actions to habitual behaviors, and vice-versa, is mutable and habits can be inhibited allowing goal-directed behaviors to be reinstated.

1.1.1. Identifying Habitual Responding Empirically

Since goal-directed responding is driven by the reinforcing value of the outcome (Adams & Dickinson, 1981), and habitual behavior is driven by environmental stimuli regardless of the motivational value of the reward (Adams, 1982), goal-directed and habitual behavior can be detected in the laboratory after a response has been learned using reinforcer devaluation (RD) procedures (Colwill and Rescorla, 1985; Yin et al., 2004; Thrailkill and Bouton, 2015; Trask et al., 2020). During these procedures, reward value of the sucrose pellet reinforcer in subjects randomly assigned to the Devalued group is extinguished through taste aversion conditioning following acquisition where the sucrose reinforcer becomes associated with injections of LiCl - which induces nausea. Devalued animals that are goal-directed when evaluated under extinction conditions are expected to decrease responding for the reinforcer now that the action is associated with an outcome that is no longer valued. Conversely, habitual behavior can be identified by a continuation of responding in the Devalued group compared to the Non-Devalued group in which the value of the outcome did not change following RD.

1.2. Brain Regions Involved in Actions and Habits

Multiple regions of the brain – such as the medial prefrontal cortex, hippocampus, amygdala, and dorsal striatum to name a few – process stimuli to produce motor outputs (Gruber and McDonald, 2012; Hogarth et al., 2013; Lingawi et al., 2018). Humans and animals rely on these pathways to process reward information and regulate instrumental learning. We have particularly been focusing on the two subregions of the dorsal striatum to elucidate how this region coordinates instrumental learning with behavioral outputs.

1.2.1. Actions or Goal-Directed Responding

The dorsal medial striatum (DMS) of the dorsal striatum has been found to regulate goal-directed outputs since an inactivation of this region by lesioning it resulted in an absence of the acquisition and expression of goal-directed behavior (Yin, Ostlund, Knowlton & Balleine, 2005). As mentioned previously, the rewarding value of an action-outcome association is essential to producing goal-directed behavior; however, the rewarding value of the association can be disrupted by interference with goal-directed processes (reward devaluation) in the laboratory.

1.2.2. Stimulus Response or Habitual Responding

The other region of the dorsal striatum that is involved in motor learning and regulates S-R associations is the dorsolateral striatum (DLS; Jog, 1999; Packard & Knowlton, 2002; Yin, Knowlton, & Balleine, 2006; Packard and Goodman, 2012; Macpherson, Morita, & Hikida, 2014; Graybiel & Grafton, 2015). Yin & Knowlton, (2006), determined this when lesions to the DLS resulted in a reinstatement of DMS control over behavior and a continuation of goal-directed responding even after reward

devaluation. More recently, however, van Elzelingen et al., (2022), found that optogenetic dopamine stimulation in the DMS – but not the DLS – accelerates habit formation and can be used to predict whether a subject will become habitual. The opposing theories of how the DMS and DLS function in mediating the transition from goal-directed actions to habits is still unclear and extensive research needs to be conducted to fully understand this mechanism. When goal-directed actions transition to habit, or vice versa, the DMS and the DLS do not completely shut down. Instead, whichever structure is more active controls the circuitry to produce the behavioral output (Turner et al., 2022), indicating that these behaviors are flexible and can be reinstated.

1.2.3. Estrogenic Effects on the Striatum

Estrogen binding to membrane estrogen receptors has been found to influence DA release in the striatum. E2 directly decreases electrical activity of MSNs to decrease GABA inhibition and indirectly increase DA release (Krentzel et al., 2019). This supports previous literature which showed that E2 infusions directly into the dorsal striatum rapidly increased striatal DA release (Shams et al., 2016). Because of E2's ability to increase DA release in the striatum, possibly specifically in the DLS (Yoest et al., 2018), it was believed that high estrogen states increased motivation for reward behaviors such as sex and drug seeking (Yoest et al., 2014). However, recent studies in our laboratory have shown that intact females who received a bolus of proestrus level E2 the night before the extinction test demonstrated goal-directed behavior when trained to 240 R-Os, an amount of training where we expect to see well-ingrained habit in females (data not yet published). Goal-directed responding in those females was the antithesis of how we would normally

characterize it (as an increased rate of responding in animals in the Non-Devalued group compared to that of subjects in the Devalued group) since we observed an increased rate of responding in the Devalued group when compared to the Non-devalued group because these were overtrained rats (data not yet published). From these observations which are supported in the literature, an inverted-U relationship of E2 on dopamine release and habit development has suggested that there is an optimal amount of DA release in the striatum to support the transition of goal-directed actions to habits or vice versa (other reports discussed in detail in Barth et al., 2015; Schoenberg et al., 2022).

1.2.4. Sex Differences in Structural Organization and Hormonal Activation

It is still unclear how the regions of the dorsal striatum regulate the transition from goal-directed actions to habits during instrumental learning in a sex-specific manner with intact females expressing habitual behavior significantly earlier in instrumental training than intact males (Schoenberg et al., 2019; Dickinson et al., 1995). Thus, structural organization and hormonal activation differences – in particular the effects of estrogen and progesterone in females and testosterone in males – between the sexes are actively being studied to determine the roles these dissimilarities play on habit development. Although structural sexual dimorphisms exist in the hippocampus, amygdala, and cortex (Cahill, 2006; Cosgrove et al., 2007; Gillies & McArthur, 2010b), sex differences in MSN neuron density have not been found, and the overall volume of the striatum does not robustly differ between males and females (Meitzen et al., 2011).

The primary goal of the present experiments was to discern if testicular hormones have an impact on habit development in male rats at all (Experiment 1), and secondarily to

begin to unravel the effects E2 specifically has on habit development in male rats (Experiment 2). Based on the inverted U-model of E2 on DA release, and the fact that males are exposed to constant high E2 aromatized from T, we hypothesize that testicular steroid hormones delay habit in intact males and thus, CAST males will express habitual behavior earlier in instrumental training than intact males (Experiment 1). We also hypothesize that estrogen replacement will restore habitual responding at a level of training when it has been seen in intact males (Experiment 2) .

CHAPTER 2: EXPERIMENT 1A-D: EFFECTS OF CASTRATION ON HABIT DEVELOPMENT IN MALE RATS

2.1. Introduction

During a critical period of prenatal development, the male and female brain grows in a vastly different hormonal environment. Males are exposed to testosterone and consequently high estrogen while females are shielded from the masculinizing effects of estrogens by the mother's placenta. This prenatal exposure leads to permanent sex differences in the organizational hard-wiring of the rat, as well as the human, brain. Not only are sexual dimorphisms found in structures of the brain such as the hippocampus, amygdala, and cortex (Cahill, 2006; Cosgrove et al., 2007; Gillies & McArthur, 2010b), the sexually imprinted brain may subsequently be influenced by the activation of circulating gonadal steroids.

Circulating gonadal hormones – estradiol and progesterone in females and testosterone (T) in males – exert their effects on brain structures, and consequently can alter behavior in a sex-specific manner (Schoenberg et al., 2019; Schoenberg et al., 2022). In addition, in contrast to the cyclic fluctuation of E2 and progesterone in intact females throughout the estrus cycle, T in males is chronically either reduced to dihydrotestosterone (DHT) by 5α-reductase, or aromatized by cytochrome P450 aromatase to E2. It is through these two steroid hormones that T promotes male sex differentiation and can exert downstream effects on behavior, and thus, male rats have constant (not cyclic) exposure to both androgens and estrogens.

In males, testosterone concentrations in the hypothalamus and blood serum significantly increase on gestation day 18 and again 2 hours postnatally (Weisz and Ward, 1980; Rhoda et al., 1984; Corbier et al., 1992). These two T surges have been found to be responsible for the differentiation of male rats in two ways: T directly masculinizes brain regions and E2 aromatized from T concurrently defeminizes the brain. E2's role in preventing feminization by promoting masculinization were determined by Rhoda et al., (1984), when administration of an aromatase inhibitor in newborn male rats resulted in behavioral feminization during adulthood.

It has previously been shown in the literature that intact male rats respond habitually when trained to at least 360 response-reinforcer pairings (Dickinson et al., 1995). However, habitual responding in intact male rats with that amount of training has not been confirmed using the present behavioral paradigm which differs from the methodology used by Dickinson et al, (1995) in the type of reward devaluation procedure employed – taste aversion conditioning versus food deprivation, respectively. Throughout the 1950s, Garcia et al. (1955, 1956, 1957), studied taste aversion and determined that taste aversions are more effective than any other type of aversion conditioning including sound or sight aversions, and this has been observed more recently by Hammerslag & Gulley, (2014) who noticed that female rats appeared less sensitive to outcome devaluation by satiety of a sucrose reward than males.

Since we have previously shown that gonadal steroid hormones impact habit development in intact female rats (Schoenberg et al., 2022), the present experiment was designed to study the effects of castration on habit development in male rats. We

specifically wanted to determine whether male gonadal hormones function in the development of habit in intact male rats and to delineate the number of reinforced exposures at which CAST males switch from goal-directed to S-R responding. In Experiment 1A, CAST males were trained to160 response-reinforcer pairings. This amount of training was chosen because that is when the transition from goal-directed actions to habits occurs in females (Schoenberg et al., 2019). Experiment 1B was conducted with CAST males trained to 240 R-Os since habitual responding was observed in CAST males trained to 320 R-Os and 400 R-Os (Experiments 1C and 1D, respectively). These training amounts take place at the beginning (320 response reinforcer-exposures) and the end (400 R-Os) of the transitional period of habit formation in intact male rats (Dickinson et al., 1995), and would indicate an early transition to habit if it is observed at 320 R-Os. With training to 400 R-Os, we expected to see well-ingrained habit in intact male rats as was observed with overtrained intact female rats (trained to 240 ROs; data not yet published), which was characterized by increased rates of responding in the Devalued group when compared to the Non-devalued group during the extinction test. However, if we did not observe indelible habit with this extensive amount of training, this would indicate that gonadal hormones eventually contribute to the expression of habit in intact males.

For Experiments 1C and 1D, immunocytochemistry (ICC) was performed to stain for post-synaptic density protein 95 (PSD-95). PSD-95 is a scaffolding protein that has been implicated in synaptic development and plasticity, and can be used to identify the density of synapses. Since an increase in synaptic density has been associated with the continuation of behavior and the formation of habit (Avchalumov et al., 2020), we

hypothesized that we would see a positive correlation between the density of PSD-95 in the dorsal striatum of Non-Devalued CAST male rats with greater amounts of training (320 R-Os and 400 R-Os, respectively) corresponding with the transition from goal-directed actions to habitual responding.

2.2. Methods

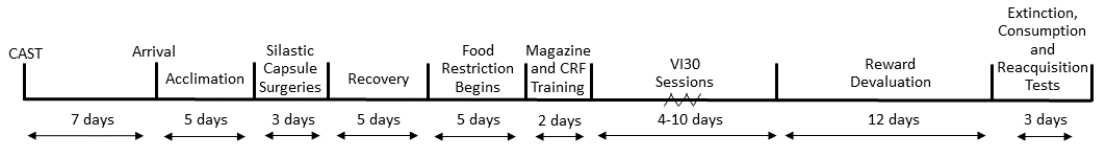

Figure 1. Experimental timeline for Experiments 1A-D and Experiment 2. In Experiments 1A-D, all animals underwent a silastic capsule implant surgery with capsules filled with 100% cholesterol. In Experiment 2, all animals had capsules with 10% 17-β estradiol and 90% cholesterol implanted. The jagged line during the VI-30 nose-poke acquisition sessions represents a variable amount of days dependent on the experiment. Nose-poke acquisition took a total of 4, 6, 8, 10, or 4 days in Experiment 1A, 1B, 1C, 1D, and 2 respectively.

2.2.1. Experimental Timeline

Each experiment took between 39 and 45 days from start to finish (Figure 1). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

2.2.2. Animals

Sixty-eight adult male Long Evans arrived to our colony room no more than 1 week after having been castrated by the vendor (Charles River, Quebec; 90 days old at the time of arrival). They 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 to the colony room to acclimate before undergoing a noninvasive surgery to implant a 10 mm porous silastic capsule containing 100% cholesterol (vehicle) subcutaneously at the scruff

of their neck (Almey et al., 2013). This was done to allow for equal treatment in any potential follow-up experiments where we may want to replace testicular hormones in CAST male rats. Following five days of recovery from surgery, all animals were put on restricted feed to maintain a target weight of 85% of their *ad libitum* weight for the duration of the experiment to ensure they were motivated to earn sucrose pellet reinforcers during training. This was done by weighing rats daily and calculating the amount of food needed to maintain their target weight.

2.2.3. Instrumental Training Apparatus

The training apparatus consisted of six standard rat operant chambers (Med Associates, St. Albans, VT) kept within individual noise-attenuating cabinets. 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 port was a nose-poke device (ENV-114, Med Associates) that emitted an infrared beam. When animals performed a nose-poke, the 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). Each rat was assigned to a specific operant chamber in which they were trained and tested for the duration of the experiment. The house light in the chambers turned on at the beginning of each session, and automatically turned off at the termination of each session.

2.2.4. Magazine Training

All rats received two 30-min sessions of magazine training, during which sucrose pellet reinforcers were presented on a variable-time 60-s (VT 60-s) schedule. During these

sessions, the nose-poke device was physically removed from the operant chambers to allow the animals to become familiar with the environment and learn the positive value of the sucrose reinforcer, as well as the sound of the sucrose pellet being delivered to the magazine.

2.2.5. Nose-Poke Acquisition

Following magazine training, all rats received two sessions of training with a continuous schedule of reinforcement, where every nose-poke response was reinforced, for a total of 25 reinforcers earned over the course of each session. During these two sessions, rats learned the association between the nose-poke response and receipt of sucrose pellet reinforcement. Animals then underwent 4 (Experiment 1A), 6 (Experiment 1B), 8 (Experiment 1C), or 10 (Experiment 1D) sessions (1 session per day for 4, 6, 8 or 10 days) of nose-poke acquisition training on a variable-interval 30-s (VI 30-s) schedule. Variable interval schedules have been shown to facilitate habit formation (Dickinson et al., 1983). These sessions terminated after each rat had earned 40 reinforcers, for a total of 160, 240, 320 or 400 response–reinforcer pairings over the course of the VI 30-s acquisition training. In Experiment 1A, 160 reinforcers were used as we have previously found that intact female rats demonstrate habitual behavior with this level of training (Schoenberg et al., 2019). In Experiment 1B, CAST males were trained to 240 R-Os since this level of training occurs between 160 R-Os and 320 R-Os. Studying the effects of CAST on animals trained to this amount would indicate where the transition of goal-directed actions to habits occurs in CAST males. In Experiment 1C, 320 reinforcers were used since intact males have been shown to respond habitually with training to 360 reinforcers (Dickinson et al., 1995),

therefore, habitual responding at this level of training would indicate early habit in CAST males. In Experiment 1D, 400 reinforcers were used because well-ingrained habitual behavior was expected with this level of training, thus an absence of habit would indicate that gonadal hormones play a role in eventually instating habitual responding in intact males.

2.2.6. Reinforcer Devaluation (RD)

Following the final session of acquisition, half of the animals were randomly assigned to a Devalued group, with their cage-mates matched to a Non-Devalued group. All rats then underwent a reinforcer devaluation paradigm that consisted of a series of two-day cycles (Thrailkill & Bouton, 2017). During each session of RD, nose-poke responses were prevented by physical removal of the nose-poke holes from the operant chambers. On odd-numbered days in this paradigm, all rats were placed in the operant chambers; however, only the Devalued group received sucrose pellets (starting with a total of 40 pellets on Day 1). On these days, rats in the Non-Devalued group did not receive any sucrose, and their sessions were time-yoked to their Devalued counterparts. Upon the completion of each session, all rats were removed from the operant chambers and injected intraperitoneally (i.p.) with a 10 ml/kg dose of 0.15 M LiCL to induce nausea, then returned to their home cages. On even-numbered days, the same procedure was conducted, but only the Non-Devalued group received sucrose reinforcers; while the Devalued group was placed in the operant chambers for the same duration as their yoked counterparts without receiving any sucrose reinforcers. 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. Using this paradigm, the following factors were counterbalanced: exposure to the operant chamber, the sucrose reinforcer, and to the same number of injections of both LiCl and saline. The only difference between the Devalued and Non-Devalued groups was that the association of the LiCl induced nausea with the sucrose reinforcer only occurred in the Devalued group. As devaluation continued, animals in the Devalued group consumed increasingly fewer pellets during their sessions on odd-numbered days. On even-numbered days, animals in the Non-Devalued group were presented with the average number of pellets consumed by the Devalued group the previous day. These two-day cycles proceeded until all animals in the Devalued group stopped consuming sucrose pellets. This criterion of zero consumption in the Devalued group is considered evidence of a successful conditioned taste aversion to sucrose reinforcers, and allows for the interpretation of responding during the extinction test to be evidence of responding in habitual behavior. RD required a total of 12 days (or six cycles) for each experiment 1A-D.

2.2.7. Testing for Habitual Behavior – Extinction Test

On the day following the last day of RD, sensitivity to RD was tested during a 12-minute session under extinction conditions where the nose-poke holes were present in the chambers, giving animals the possibility to respond, however, responding did not lead to reinforcement. Nose-pokes per minute were recorded by the computer software. If nose-poking was still a goal-directed action, it was expected that the Devalued group would respond significantly less than the Non-Devalued group. If nose-poking was insensitive to RD and therefore habitual, it was expected that rats in the Devalued group would continue nose-poking at a rate equivalent to that of the Non-Devalued group.

2.2.8. Confirming Successful Taste Aversion Conditioning – Consumption Test

On the day following the extinction test, successful devaluation of the sucrose reinforcer was assessed during a brief consumption test. During this test, 10 sucrose pellets were delivered on a VT 30-s schedule. Nose-pokes were again physically removed, and consumption of sucrose pellets for each animal was recorded. It was expected that animals in the Devalued group would associate the delivered sucrose pellet with nausea and would avoid consuming any sucrose pellets - evidence of successful taste aversion conditioning. Contrarily, animals in the Non-Devalued group were expected to consume all delivered pellets. Animals in the Devalued group who failed to demonstrate successful taste aversion conditioning (consumed five or more pellets during the consumption test) were excluded from all analyses.

2.2.9. Confirming Successful Taste Aversion Conditioning – Reacquisition Test

On the day following the consumption test, successful devaluation was assessed again during a 30-minute reacquisition test. Rats were placed in the operant boxes and again allowed to nose-poke for the reinforcer, which was delivered on a VI 30-s schedule, similarly to the initial acquisition sessions. During the reacquisition test, the Devalued group should integrate the memory of the conditioned taste aversion to sucrose with the memory that the nose-poking response earns reinforcers. The Devalued group was, therefore, expected to exhibit decreased nose-poking (or a failure to reacquire) for the sucrose reinforcer as evidence of successful taste aversion conditioning. On the other hand, Non-Devalued groups, for whom the reinforcer maintains its value throughout RD, were expected to reacquire the nose-poke behavior to baseline levels of responding.

2.2.10. Statistical Analyses

Average response rates (nose pokes) per minute in acquisition were analyzed using repeated-measures ANOVA with a within-subjects factor of training session and between-subjects factor of anticipated devaluation group (Non-Devalued or Devalued). Future devaluation group was included to ensure that there were no baseline differences in acquisition response rates. Greenhouse-Geisser ($\varepsilon < 0.75$) or Huynh-Feldt ($\varepsilon > 0.75$) corrections of degrees of freedom were utilized in cases where sphericity was violated (Mauchly's test $p < 0.05$). Response rates during the critical test in extinction and reacquisition were analyzed as a proportion of baseline response rates in an independent samples *t*-test. Baseline response rates are response rates at test divided by response rates from the final session of acquisition (Nelson & Killcross, 2013; Shipman et al., 2018, Schoenberg et al., 2021). Outlier performance at test was operationalized as two standard deviations above or below the group mean ($z = \pm 2$; Field, 2007), and these animals were excluded from all analyses. Criterion for significance for all tests was set at $p < 0.05$.

2.2.11. Immunocytochemistry

A total of 8 animals from experiments 1B and 1C – 4 Non-Devalued trained to 320 reinforcers and 4 Non-Devalued trained to 400 reinforcers – were deeply anesthetized with isoflurane and transcardially perfused with ice-cold 0.9% physiological saline followed by 4% paraformaldehyde. Only Non-Devalued subjects were used for ICC since reward devaluation may have impacted Devalued subject brains resulting in confounding effects outside of training amount. Brains were extracted and allowed to fix in 4% paraformaldehyde for 24-h followed by 30% sucrose for 72-h for cryoprotection. Once

brains were cryoprotected, they were frozen in optimal cutting temperature solution, and sliced at 40 μm on a Leica CM1950 cryostat (Perkins Biomedical Services). Four replicate vials of coronal brain sections throughout the dorsal striatum (collection commenced just anterior and concluded just posterior to the dorsal striatum, Paxinos & Watson, 1998) for each subject were stored in vials containing 0.1 M phosphate-buffered saline (PBS) at 4 °C. At least one replicate per subject was stained as experimental sections, and at least one replicate per animal was stained as control slices. Prior to blocking in 0.1 M PBS; 0.1% BSA; 0.2% Triton X-100; 2% goat serum for 1.5-h, experimental slices were washed four times with 0.1 M PBS at room temperature. The slices were then incubated at 4 °C overnight in primary antibody PSD-95 rabbit antiserum (1:1000; Abcam ab18258). Control slices underwent the same staining procedure; however, they were incubated overnight at 4 °C in blocking solution instead of primary antibody PSD-95. The following day, experimental and control sections were washed thoroughly (seven times) with 0.1 M PBS then incubated with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:500, catalog #A32731; Thermo Fisher Scientific, Inc.) for 1.5-h at room temperature. Brain slices were washed four more times, then mounted in order (most anterior to most posterior slice) on SuperFrostPlus slides (Thermo Fisher Scientific, Inc.) and cover-slipped with VECTASHIELD Antifade mounting medium (Vector Laboratories). Slides were imaged using a Zeiss Axioskop 50 fluorescent microscope to visualize PSD-95 staining. Images were captured using Stereo Investigator software (MBF Bioscience, Williston, VT, USA). Quantification of PSD-95 was meant to be used to compare marker density (spots/μm^3) in animals trained to different R-O exposures.

2.3. Results

2.3.1. Experiment 1A

Acquisition data were analyzed using a repeated-measures ANOVA with a within-subjects factor of training session (4); and a between-subjects factor of anticipated devaluation groups. Mauchly's test indicated a violation of the assumption of sphericity ($\chi^2(5) = 28.37$, $p < 0.001$) resulting in a correction of the degrees of freedom with Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.45$). Results indicated that there was a significant main effect of session during acquisition ($F(1.35, 17.52) = 22.75$, $p < 0.001$), confirming that all animals acquired the behavior of nose-poking for sucrose reinforcers. There was no main effect of anticipated devaluation group (F's ≤ 1), indicating that there were no baseline differences in acquisition rates regardless of whether they were assigned to the Non-Devalued or Devalued groups for RD. Mean responses per minute during each acquisition session can be seen in Figure 2A.

All subjects in the Devalued condition reached criterion of zero consumption of pellets by the end of RD training (see Figure 2B). All subjects in the Non-Devalued condition consumed all delivered pellets on even numbered days of RD sessions (data not shown). RD took six cycles, or a total of twelve days of alternating LiCl and saline injections.

One animal exhibited outlier performance at test and was excluded from all analyses ($z = 2.13$). The final n in each group is represented in Figure 2C. Results from an independent-samples t-test demonstrated that response rates in the Non-Devalued group ($M = 0.29$, $SD = 0.07$) were significantly higher than response rates in the Devalued group

(M = 0.18, SD = 0.05; t(12.6) = 3.6, p = 0.003, Hedges' g = 0.07), indicating that CAST rats trained to 160 R-O exposures remained goal-directed (see Figure 2C).

Two animals in the Devalued group consumed more than five sucrose pellets during the consumption test, failing to demonstrate a successful taste aversion to the sucrose reinforcer, and were therefore excluded from all analyses. All other subjects in the Devalued group ate an average of zero sucrose pellets during the consumption and reacquisition tests while all animals in the Non-Devalued group consumed all delivered pellets as expected. Results from the reacquisition test indicate that subjects in the Non-Devalued group reacquire the nose-poke behavior to baseline levels of responding (M = 1.12, SD = 0.19), while animals in the Devalued condition failed to reacquire (M = 0.17, SD = 0.05). There was a significant difference between response rates of subjects in the Non-Devalued group and Devalued group (t(9.57) = 14.14, p < 0.001, Hedge's g = 0.16, see Figure 2D).

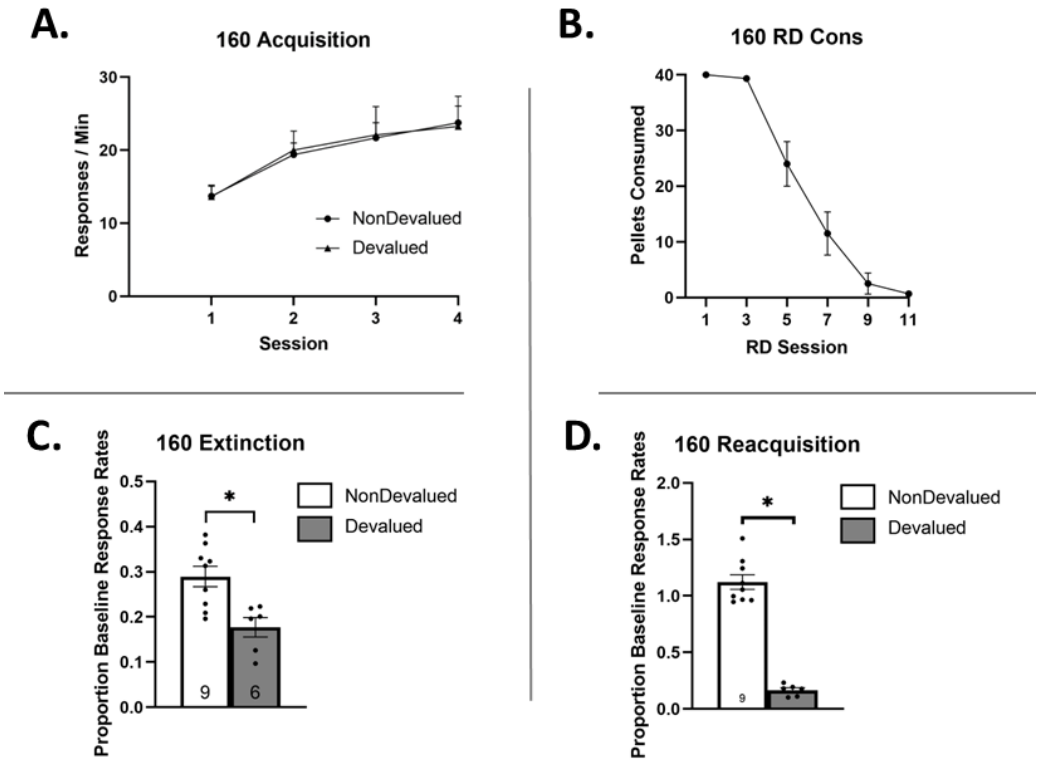

Figure 2A-D. A. Average responses per minute across four VI 30-s sessions differentiated by future devaluation groups. B. Mean number of sucrose pellets consumed by Devalued animals during RD which took six cycles (12 days). C. Mean responses per minute by the Devalued and Non-Devalued groups during the extinction test as a proportion of baseline. Numbers in bars represent final group n's. D. Mean responses per minute during the reacquisition test by Devalued and Non-Devalued groups as a proportion of baseline. Error bars represent SEM.

2.3.2. Experiment 1B

All subjects acquired the nose-poking behavior as seen in Figure 3A by an increase in response rates over the course of acquisition which was a total of six sessions in this experiment. Mauchly's test was significant ($\chi^2(14) = 37.1$, $p < 0.001$), therefore Greenhouse-Geisser correction of degrees of freedom was used ($\varepsilon = 0.49$). Repeated-measures ANOVA confirmed there was a significant main effect of session ($F(2.44, 39.05) = 34.88$, $p < 0.001$) but no main effect of future devaluation group during acquisition (F's ≤ 1).

All subjects in the Devalued condition acquired the taste aversion to the sucrose reinforcer and reached criterion of zero consumption of pellets by the end of RD training (see Figure 3B). All subjects in the Non-Devalued condition consumed all delivered pellets during RD sessions (data not shown). RD took six cycles, or a total of twelve days of alternating LiCl and saline injections.

Analysis by an independent-samples *t*-test indicated no significant difference between response rates in the Devalued group ($M = 0.26$, $SD = 0.08$) and the Non-Devalued group ($M = 0.22$, $SD = 0.09$; $t(16) = 1.00$, $p = 0.33$, Cohen's $d = 0.08$), indicating that CAST rats trained to 240 R-O exposures expressed habitual behavior (see Figure 3C).

In this experiment, all subjects in the Devalued group showed a complete taste aversion during the consumption and reacquisition tests. All Non-Devalued animals consumed all delivered pellets during both tests as expected. There was a significant difference between response rates of subjects in the Non-Devalued group ($M = 1.07$, $SD = 0.22$) and Devalued group ($M = 0.18$, $SD = 0.06$, $t(9.33) = 11.48$, $p < 0.001$, Hedge's $g = 0.17$, see Figure 3D), indicating that Non-Devalued subjects reacquired to baseline levels of responding while animals in the Devalued condition failed to reacquire.

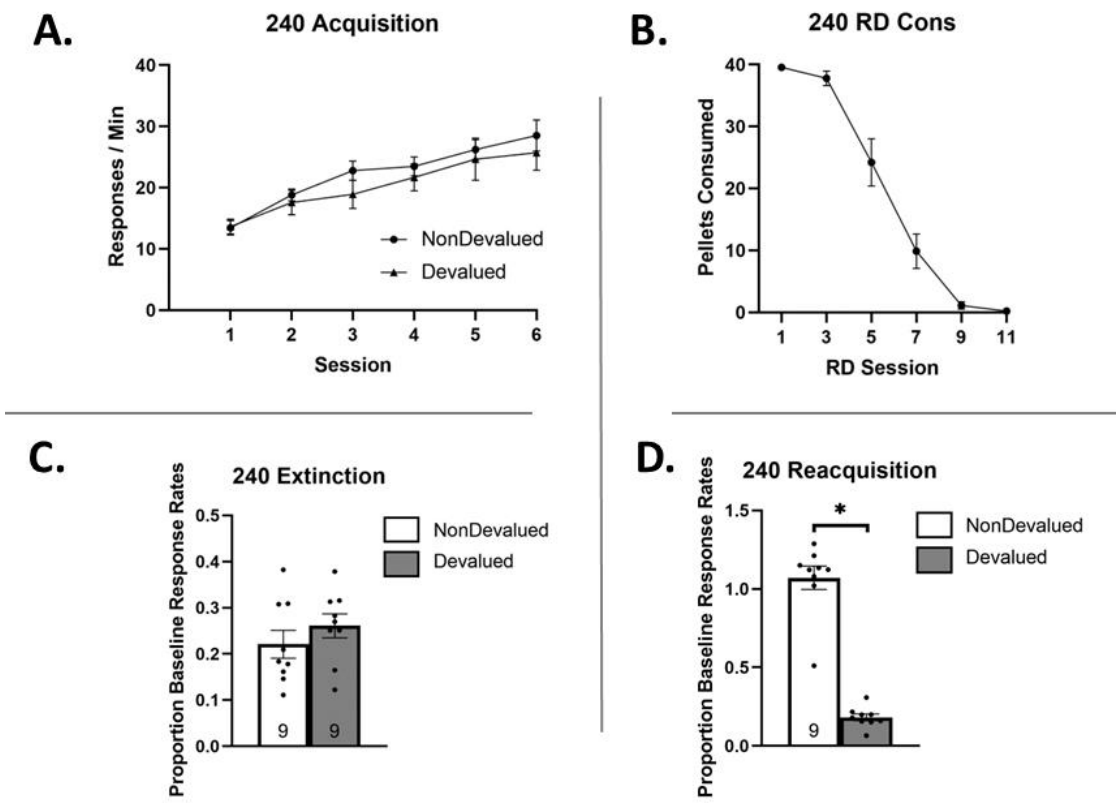

Figure 3A-D. A. Average responses per minute across six VI 30-s sessions differentiated by future devaluation groups. B. Mean number of sucrose pellets consumed by Devalued animals during RD which took six cycles (12 days). C. Mean responses per minute by the Devalued and Non-Devalued groups during the extinction test as a proportion of baseline. Numbers in bars represent final group n's. D. Mean responses per minute during the reacquisition test by Devalued and Non-Devalued groups as a proportion of baseline. Error bars represent SEM.

2.3.3. Experiment 1C

Repeated-measures ANOVA indicated that there was a significant main effect of session ($F(2.28, 34.14) = 42.26$, $p < 0.001$, Mauchly's test was significant ($\chi^2(27) = 97.48$, $p < 0.001$), therefore Greenhouse-Geisser correction of degrees of freedom was used ($\varepsilon = 0.33$). This analysis showed that all subjects acquired the nose-poking over the course of eight acquisition sessions (see Figure 4A). Results also demonstrated that there was no main effect of future devaluation group during acquisition (F's ≤ 1).

All subjects in the Devalued group reached criterion of zero consumption of pellets by the end of RD training (see Figure 4B). All Non-Devalued subjects consumed all delivered pellets during RD sessions (data not shown). RD took six cycles, or a total of twelve days of alternating LiCl and saline injections.

Results of an independent-samples *t*-test demonstrated that CAST rats trained to 320 R-O exposures express habitual behavior. There was no significant difference between response rates under extinction conditions in the Devalued group ($M = 0.13$, $SD = 0.06$) and the Non-Devalued group ($M = 0.17$, $SD = 0.07$; $t(15) = 1.35$, $p = 0.19$, Cohen's $d = 0.07$, see Figure 4C).

One animal in the Devalued condition failed to develop a complete taste aversion to the sucrose pellet reinforcers and consumed more than five pellets during the consumption test. This animal was, therefore, removed from all analyses. All other subjects in the Devalued group showed a complete taste aversion during the consumption and reacquisition tests. Conversely, all Non-Devalued animals consumed all delivered pellets during both tests as expected. Non-Devalued subjects reacquired to baseline levels of responding while animals in the Devalued condition failed to reacquire (see Figure 4D), as evidenced by the significant difference between response rates of subjects in the Non-Devalued group ($M = 0.91$, $SD = 0.26$) and Devalued group ($M = 0.10$, $SD = 0.06$, $t(9) = 9$, $p < 0.001$, Hedge's $g = 0.21$).

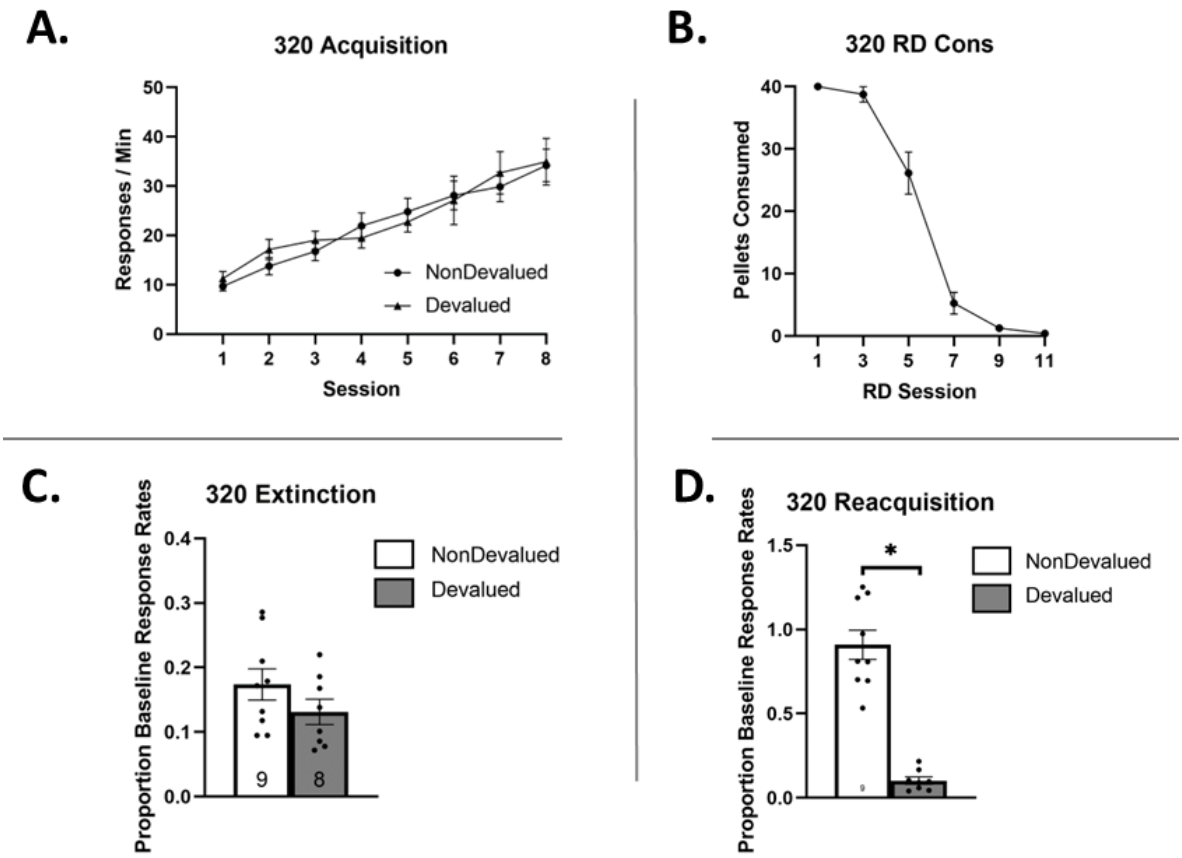

Figure 4A-D. A. Average responses per minute across six VI 30-s sessions differentiated by future devaluation groups. B. Mean number of sucrose pellets consumed by Devalued animals during RD which took six cycles (12 days). C. Mean responses per minute by the Devalued and Non-Devalued groups during the extinction test as a proportion of baseline. Numbers in bars represent final group n's. D. Mean responses per minute during the reacquisition test by Devalued and Non-Devalued groups as a proportion of baseline. Error bars represent SEM.

Immunofluorescent analysis of PSD-95 in brain cells of Non-Devalued CAST male rats trained to 360 R-O exposures. Images, which were taken at 20X magnification, indicate that staining protocol was effective for visualization of PSD-95 (bright lime green spots) and secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (yellowish diffuse background staining). However, quantification of PSD-95 could not be conducted since brain tissue quality was not appropriate to distinguish striatal striosomes from other cells and we did not stain cell nucleii with DAPI.

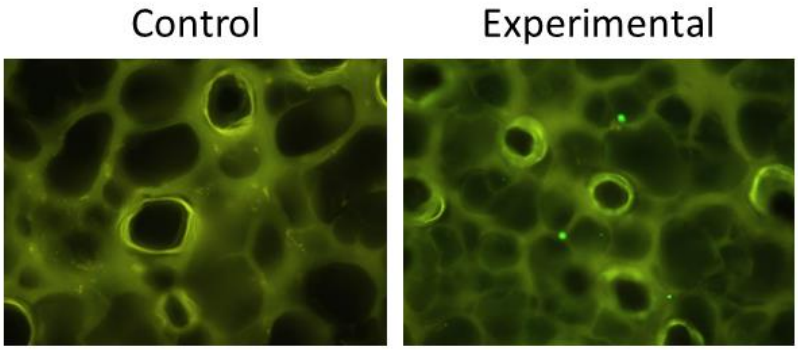

Figure 5. Immunofluorescent staining for PSD-95 in brain cells of Non-Devalued CAST males trained to 360 R-O exposures (20X magnification). Experimental brain slices showed lime green positive staining for PSD-95 compared with control slices which were only stained with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG.

2.3.4. Experiment 1D

All animals acquired the behavior as indicated by an increase in response rates over the course of the 10-session acquisition training (see Figure 6A). Repeated-measures ANOVA showed Mauchly's test was significant ($\chi 2(44) = 72.45$, $p = 0.008$), therefore Greenhouse-Geisser correction of degrees of freedom was used ($\varepsilon = 0.42$). There was a significant main effect of session ($F(3.76, 56.43) = 70.72$, $p < 0.001$), but no main effect of future devaluation group during acquisition (F's ≤ 1).

All subjects in the Devalued group reached criterion of zero consumption of pellets by the end of RD training (see Figure 6B). All Non-Devalued subjects consumed all delivered pellets during RD sessions (data not shown). RD took six cycles, or a total of twelve days of alternating LiCl and saline injections.

One subject in the Non-Devalued condition exhibited outlier performance at test and was excluded from all analyses ($z = 2.09$). Results of an independent-samples *t*-test demonstrated that there was no significant difference between response rates in the Devalued group ($M = 0.12$, $SD = 0.06$) and the Non-Devalued group ($M = 0.16$, $SD = 0.04$;

$t(15) = 1.56$, $p = 0.14$, Cohen's $d = 0.06$, see Figure 6C) during the extinction test. This shows that CAST rats trained to 400 R-O exposures express habitual behavior.

All subjects in the Devalued group showed a complete taste aversion during the consumption and reacquisition tests. All Non-Devalued animals consumed all delivered pellets during both tests as expected. Non-Devalued subjects reacquired to baseline levels of responding while animals in the Devalued condition failed to reacquire as evidenced by the significant difference between response rates of subjects in the Non-Devalued group ($M = 0.95$, $SD = 0.29$) and Devalued group ($M = 0.08$, $SD = 0.05$, $t(7.31) = 8.23$, $p < 0.001$, Hedge's $g = 0.22$, see Figure 6D).

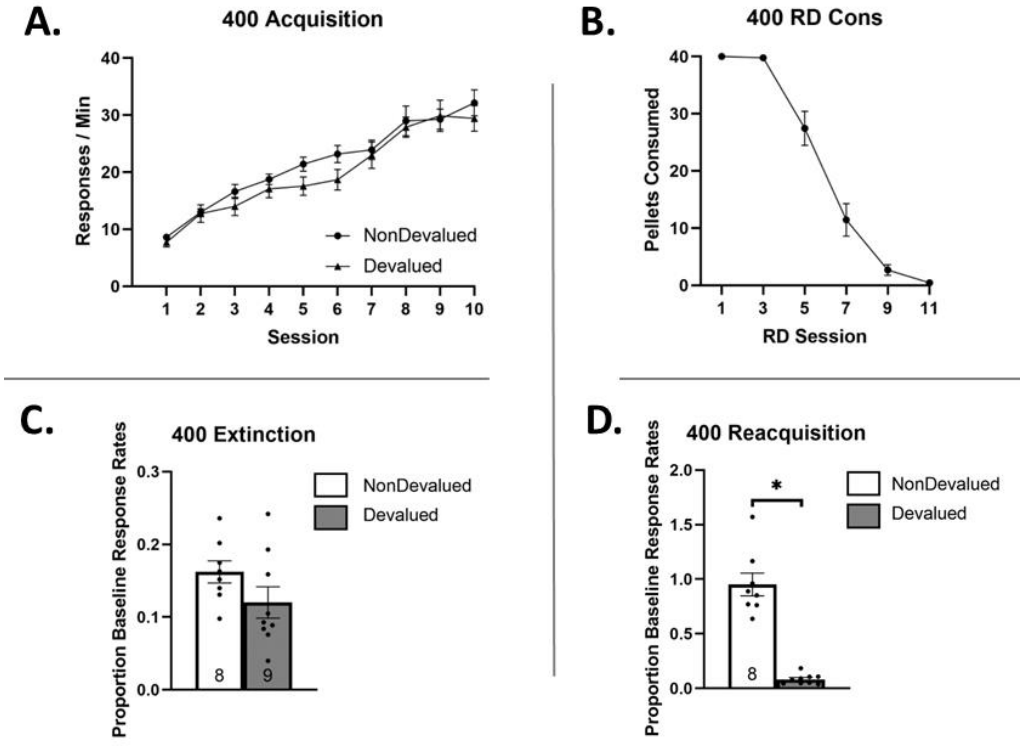

Figure 6A-D. A. Average responses per minute across six VI 30-s sessions differentiated by future devaluation groups. B. Mean number of sucrose pellets consumed by Devalued animals during RD which took six cycles (12 days). C. Mean responses per minute by the Devalued and Non-Devalued groups during the extinction test as a proportion of baseline. Numbers in bars represent final group n's. D. Mean responses per minute during the reacquisition test by Devalued and Non-Devalued groups as a proportion of baseline. Error bars represent SEM.

Immunofluorescent analysis of PSD-95 in brain cells of Non-Devalued CAST male rats trained to 400 R-O exposures. Images, which were taken at 20X magnification, indicate that staining protocol was effective for visualization of PSD-95 (bright lime green spot) and secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (pale yellow-green diffuse background staining). However, quantification of PSD-95 could not be conducted since brain tissue quality was not appropriate to distinguish striatal striosomes from other cells and we did not stain cell nucleii with DAPI.

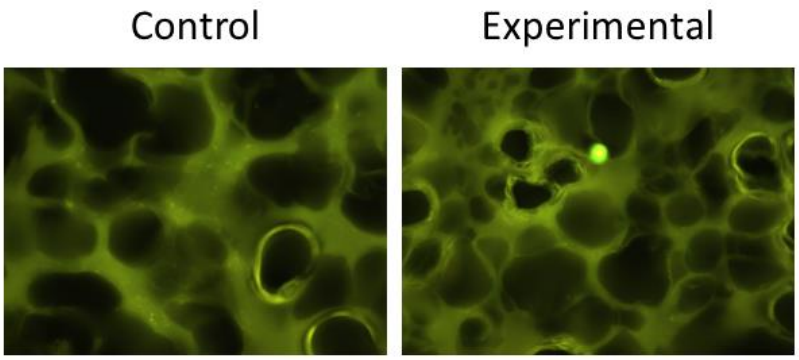

Figure 7. Immunofluorescent staining for PSD-95 in brain cells of Non-Devalued CAST males trained to 400 R-O exposures (20X magnification). Experimental brain slices showed lime green positive staining for PSD-95 compared with control slices which were only stained with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG.

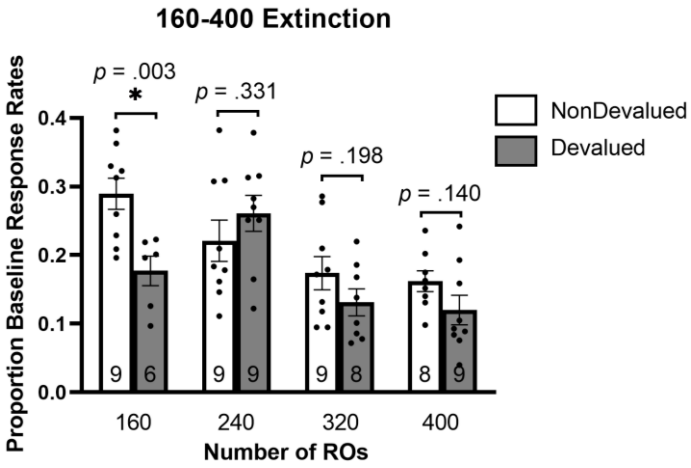

Figure 8. Compiled response rates during the extinction tests with different amounts of training as a proportion of baseline. With training to 160 R-Os, the Devalued group responded significantly lower than the Non-Devalued group (p = 0.003). At the other training conditions, there was no significant difference

between Non-Devalued and Devalued responding. Numbers in bars represent final group n's. Error bars represent SEM.

2.4. Discussion

The results from these experiments demonstrate that following a level of instrumental training (240 reinforcers) where intact males respond in a goal-directed manner (Schoenberg et al., 2019), CAST males express habitual behavior (as seen by the equivalent rates of responding in the 240 Devalued group compared to the 240 Non-Devalued group; Figure 3C). These results indicate that the transition from goal-directed actions to habits in CAST male rats occurs between 160 R-Os and 240 R-Os (Figure 8) – significantly earlier in instrumental training than it does in intact males tested in previous studies (Schoenberg et al., 2022). Since these experiments were on CAST males without hormone replacement, these data show that male gonadal steroid hormones (T, E2, or DHT, or perhaps a combination of these) are in fact playing a role in delaying habit development in intact male rats which may contribute to the observed sex difference in habit development.

Interestingly, it looks like there was a decrease in responding in the Non-Devalued groups with increased training (Figure 8), indicating that goal-directed actions may be actively suppressed in these groups, as has been observed in overtrained intact male rats (Coutereau and Killcross, 2003). However, because these CAST males were never run together in the same experiment, direct comparisons between results of these experiments cannot be conducted. It is worth noting that a decrease in the response of subjects in the non-devalued control groups is different from what we normally see with habits because

habitual responding is usually classified as an *increase* in responding despite reward devaluation due to RD-insensitivity.

The only other time we have observed a significant reduction in responding in the Non-Devalued groups compared to the Devalued groups was in a study conducted on ovariectomized (OVX) female rats who had cyclic E2+P replacement which only occurred during acquisition (Schoenberg et al., 2022), which we predict is the effect that the intact infralimbic cortex (IL) of the medial prefrontal cortex exerts on both the acquisition and expression of habitual reward-seeking behavior by actively suppressing goal-directed behavior to encourage S-R learning (Coutureau and Killcross, 2003; Haddon and Killcross, 2011; Barker et al., 2014). The early transition to habit via the potential suppression of goal-directed responding that we observed in these experiments in CAST males may be due to the effects of the removal of androgens by castration on the IL. Similarly to the striatum, the IL is heavily influenced by circulating E2 and consequently DA release (Barker et al., 2014), thus future experiments outside the scope of the present research need to be conducted to determine how the IL regulates habit development in male rats.

Results from ICC staining indicate that our protocol was effective for the visualization of PSD-95 in experimental brain slices compared to control slices (Figures 5 and 7). While approximately half of the published articles that have used the same PSD-95 antibody that was employed in these experiments used it for Western Blots, the other half used it for ICC staining. Figures from published articles that used this antibody for ICC staining indicate that our identification of bright distinct spots as positive staining for PSD-95 versus the diffuse background staining of the secondary antibody are comparable (Guo

et al., 2021; Jeckel et al., 2021). However, throughout the perfusion process, several factors can lead to damaged tissue which may be inadequate for visualization and quantification of desired proteins during ICC. One of these factors involves the incorrect placement of the needle in the apex of the heart. This can prevent the PFA from fully circulating throughout the brain which can result in a poor fix. Improper fixation can cause blood to remain in the brain resulting in the imaging of blood cells instead of brain cells.

Due to the faulty tissue quality, I was not able to quantify PSD-95 in the striatum in order to compare synaptic marker density in the slices of subjects trained to 360 R-O exposures and subjects trained to 400 R-O exposures. It would have been interesting to perform ICC using the same protocol on the Non-Devalued CAST animals trained to 160 R-Os and 240 R-Os for comparison of PSD-95 density since these training amounts occur just prior to and following the transition from goal-directed to habitual responding in CAST male rats (Figure 8), thus I predict we would observe the greatest amount of PSD-95 fluorescence in Non-Devalued CAST animals trained to 240 R-Os compared to those trained to 160 R-Os.

Ultimately, results from these experiments indicate that male gonadal steroid hormones function in delaying habit development in intact male rats since early habit was observed in CAST males. These new data demonstrate that further research needs to be conducted to elucidate exactly how male gonadal hormones regulate the transition from goal-directed actions to habitual responding in male rats, and fueled the subsequent experiment presented here which describes the initial puzzle piece to studying how E2 specifically impacts habit formation.

CHAPTER 3: EXPERIMENT 2: EFFECTS OF ESTRADIOL REPLACEMENT ON EARLY HABIT IN CASTRATED RATS

3.1. Introduction

Numerous studies on the effects of gonadal steroid hormones have implicated estrogens in producing a myriad of outcomes on motivation (Yoest et al., 2014), memory (Hussain et al., 2016), and its protective actions (Sherwin, 2003). These studies have primarily been conducted in female subjects while there is mounting evidence that estrogen may have opposing effects in males (Gillies and McArthur, 2010b; Schoenberg et al., 2022) due to organizational dimorphisms.

In females, high estrogen favors hippocampal-place learning at the expense of DLS mediated S-R learning (Korol and Wang, 2018). Because of E2's effects on spatial strategy selection in females, T may also have activational effects since E2 in males originates from aromatized T. This was confirmed by Spritzer et al., (2013), who discovered that low testosterone biased males toward the use of S-R strategies. However, unlike the cyclic gonadal hormone fluctuation seen in females, T is secreted chronically and is constantly being aromatized to E2, resulting in greater concentrations of E2 in the hippocampus of male rats (8 nM) than females (0.6-4.3 nM; Kato et al., 2013). While these concentrations were measured specifically in the hippocampus, and more research needs to be conducted to quantify the concentrations of E2 in the striatum, E2 functions throughout the brain of both sexes (Simerly et al., 1990).

In an early study conducted on monkeys, a sex difference in learning abilities was observed such that male monkeys learned more slowly than females, and it was reasoned

that testosterone might be responsible for this difference since higher T levels correlated with poorer learning scores (Hagger and Bachevalier, 1991). More recently, habit drinking of alcohol was found to be correlated with lower testosterone levels (Fukuhara, 2017). In all of these cases, low T – and therefore low E2 – are thought to have resulted in early learning and habit in males.

As mentioned in the main introduction, E2's influences on the striatum in females are mediated by its ability to rapidly increase DA release and turnover (Shams et al., 2016; Di Paolo et al., 1985) by binding to both membrane and nuclear estrogen receptors on dopaminergic neurons. In support of this, early literature demonstrates that testosterone (probably by way of aromatization to E2) significantly enhances activity of the dopaminergic system in adult rats which was categorized by increased dopamine transporter binding densities in the striatum (Mitchell and Stewart, 1989), and facilitates the release of DA causing increased DA in the intercellular space (de Souza et al., 2009).

Recently in our laboratory, and similarly to the hypothesized effects of high E2 in delaying habit formation in males, high E2 replacement alone during acquisition in OVX female rats was found to delay habit development when trained to 160 ROs (Schoenberg et al., 2022) – an amount of training where intact females have been shown to express habitual behavior (Schoenberg et al., 2019). These results indicated that E2 alone likely does not play a role in the early habit seen in intact females (Schoenberg et al., 2022). Based on these results from the literature, and as previously described, E2's effects on DA release in the striatum, we hypothesize that high E2 levels – an amount causing greater

than optimal DA release – may be preventing habit and maintaining goal-directed responding in intact males compared to females.

By removing endogenous T by castration and replacing the E2 being aromatized from T with equivalent levels of constant E2 in subcutaneous porous silastic capsule implants (10% 17-β estradiol and 90% cholesterol), we can determine how constant E2 from T is influencing habit formation in male rats. Based on E2's effects on delaying habit development in E2-replaced OVX females at 160 R-Os, a point in training where intact females express habitual behavior (Schoenberg et al., 2022), we hypothesize that greater than optimal for the transition from goal-directed responding to habitual responding DA levels in males caused by chronic high E2 concentrations are preventing habit development in intact males. In this experiment, 160 R-Os were used as this is the point where the transition from goal-directed actions to habits occurs in intact female rats (Schoenberg et al., 2019), however, the present experiment is just the tip of the iceberg in elucidating E2's impacts on habit development in male rats. Future experiments using a similar paradigm but greater amounts of R-O exposures will also be needed.

3.2. Methods

3.2.1. Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont. A total of 18 adult male Long Evans rats arrived to our colony room no more than 1 week after having been castrated by the vendor (Charles River, Quebec; 90 days old at the time of arrival) 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 to the colony room to acclimate before undergoing a noninvasive surgery to implant a 10 mm porous silastic capsule containing 10% 17-β estradiol and 90% cholesterol (vehicle) subcutaneously at the scruff of their neck (Almey et al., 2013). These capsules have been shown to release a steady circulating concentration of proestrus level E2 in females which is comparable to the constant circulating concentration of E2 in intact males (Mannino et al., 2005). Following five days of recovery from surgery, all animals were put on restricted feed to maintain a target weight of 85% of their *ad libitum* weight for the duration of the experiment to ensure they were motivated to earn sucrose pellet reinforcers during training. This was done by weighing rats daily and calculating the amount of food needed to maintain their target weight.

3.2.2. Instrumental Training Apparatus

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

3.2.3. Magazine Training

Magazine training was the same as in Experiment 1

3.2.4. Nose-Poke Acquisition

Following magazine training, all rats received two sessions of training with a continuous schedule of reinforcement, where every nose-poke response was reinforced, for a total of 25 reinforcers earned over the course of each session. During these two sessions, rats learned the association between the nose-poke response and receipt of sucrose pellet reinforcement. Following these sessions were four 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 160 R-O exposures over the course of training.

3.2.5. Reward Devaluation

The RD protocol for this experiment was the same as in Experiment 1.

3.2.6. Testing for Habit and Confirming Devaluation

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

3.3. Results

A 4 (training session) by 2 (devaluation group; Devalued, Non-Devalued) repeated measures ANOVA was run to examine acquisition for nose-poking for sucrose in E2 replaced CAST males. Mauchly's test indicated a violation of the assumption of sphericity ($\chi^2(5) = 30.62$, $p < 0.001$), therefore, Greenhouse-Geisser estimates of sphericity was employed to correct the degrees of freedom ($\varepsilon = 0.45$). Results indicated that all animals acquired the behavior of nose-poking for sucrose reinforcers since there was a significant main effect of session during acquisition ($F(1.39, 22.27) = 34.00$, $p < 0.001$). There was no main effect of anticipated devaluation group (F's ≤ 1), indicating that there were no baseline differences in acquisition rates. Mean responses per minute during each acquisition session can be seen in Figure 9A.

All subjects in the Devalued condition acquired the taste aversion to the sucrose reinforcer and reached criterion of zero consumption of pellets by the end of RD training (see Figure 9B). All subjects in the Non-Devalued condition consumed all delivered pellets

during RD sessions (data not shown). RD took six cycles, or a total of twelve days of alternating LiCl and saline injections.

Analysis by an independent-samples *t*-test indicated a significant difference between response rates in the Devalued group ($M = 0.13$, $SD = 0.05$) and the Non-Devalued group ($M = 0.20$, $SD = 0.06$; $t(16) = 3.02$, $p = 0.009$, Cohen's $d = 0.06$), indicating that CAST rats with E2 replacement trained to 160 R-O exposures expressed goal-directed behavior (see Figure 9C).

In this experiment, all subjects in the Devalued group showed a complete taste aversion during the consumption and reacquisition tests. All Non-Devalued animals consumed all delivered pellets during both tests as expected. There was a significant difference between response rates of subjects in the Non-Devalued group ($M = 1.10$, $SD = 0.22$) and Devalued group ($M = 0.12$, $SD = 0.05$, $t(8.68) = 12.89$, $p < 0.001$, Hedge's $g = 0.17$, see Figure 9D), indicating that Non-Devalued subjects reacquired to baseline levels of responding while animals in the Devalued condition failed to reacquire the nose-poke behavior.

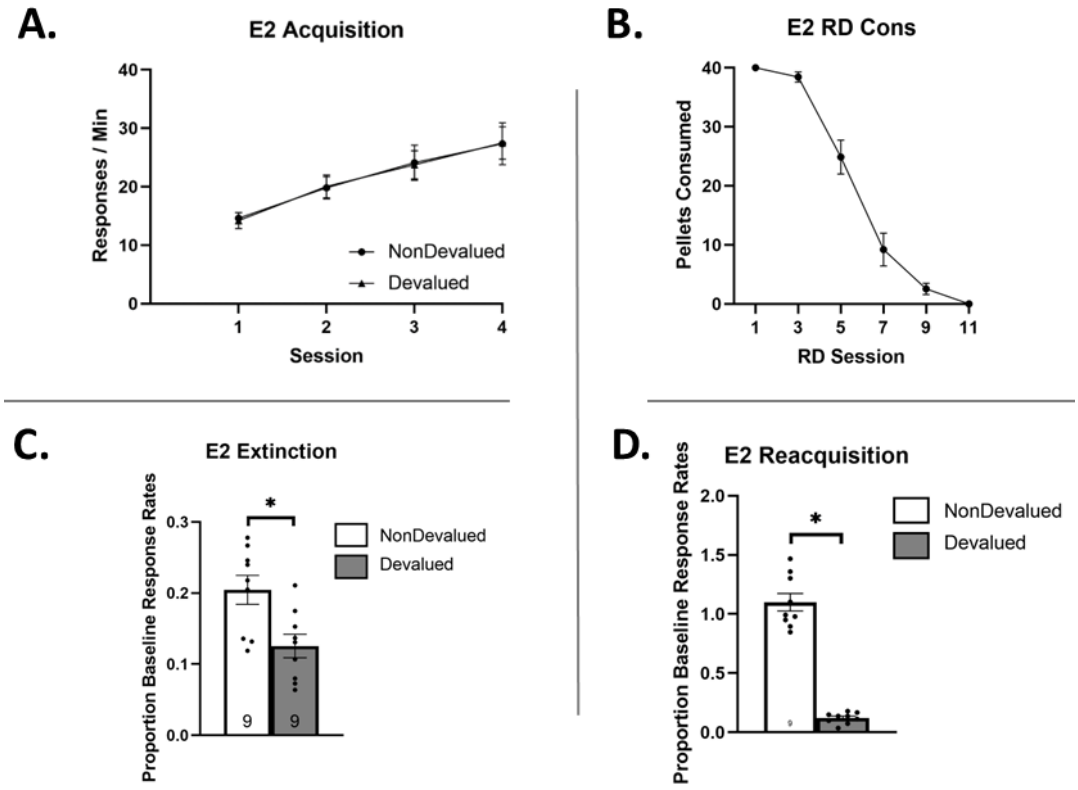

Figure 9A-D. A. Average responses per minute across four VI 30-s sessions (160 R-Os) differentiated by future devaluation groups. B. Mean number of sucrose pellets consumed by Devalued animals during RD which took six cycles (12 days). C. Mean responses per minute by the Devalued and Non-Devalued groups during the extinction test as a proportion of baseline. Numbers in bars represent final group n's. D. Mean responses per minute during the reacquisition test by Devalued and Non-Devalued groups as a proportion of baseline. Error bars represent SEM.

3.4. Discussion

At 160 reinforced exposures, E2-replaced CAST male rats express goal-directed behavior (Figure 9C). The present results did not surprise us since habit was not seen in CAST males trained to the same number of R-Os either (Experiment 1A). These data are not sufficient to conclude the effects of E2 on habit development in male rats since habitual responding is seen much later in instrumental learning in intact males (Dickinson et al., 1995). In this experiment, 160 R-Os were used as this is the point where the transition from goal-directed actions to habits occurs in intact female rats (Schoenberg et al., 2019), however, by replacing with E2 we did not simply transform CAST males into females since

organizational differences in the brain take root earlier during development and the males used in this experiment were castrated as adults.

It is important to note that in 1991, Hagger and Bachevalier noticed a reversal in the sex difference they had previously observed in habit development in neonatal monkeys. In this study, CAST male monkeys learned visual discriminations more quickly than intact males, and OVX females with androgen replacement learned more slowly than intact females (Hagger and Bachevalier, 1991). These steroid hormone manipulations occurred prior to adolescence when effects of steroid hormones have shifted from organizational to activational, and therefore, cannot be compared to our results since castration and hormone replacement occurred during adulthood in our studies.

As mentioned previously, the same experiment would need to be replicated with greater amounts of training to determine if E2 replacement in CAST males rescues the development of habit at a level of training where it is seen in intact males (360 R-Os; Dickinson et al., 1995). If it does, it would indicate that E2 is responsible for the transition from goal-directed actions to habits in intact males.

CHAPTER 4: GENERAL DISCUSSION

Novel data from these experiments demonstrate that early habit was seen in CAST males (Experiment 1, Figure 8), compared to what has previously been observed in intact males. These data indicate that male gonadal steroid hormones play a role in delaying habit in intact male rats. In addition, results from Experiment 2 reveal that training to 160 R-Os is not sufficient to produce habitual responding in E2 replaced CAST males (Experiment 2, Figure 9C), and future experiments will need to be conducted to determine exactly how E2 impacts habit development in male rats. Results from the present experiments signify that additional research must be conducted to determine precisely how male sex hormones function in delaying habit development in intact male rats compared to intact female rats.

The influences gonadal steroid hormones play in sexual behavior have been extensively studied (Phoenix et al., 1959; Whalen and Edwards, 1967), however, these hormones also impact learning, motivated behavior, sensorimotor outputs, and the development of habit (Hagger and Bachevalier, 1991; Spritzer et al., 2013; Fukuhara et al., 2017; Li et al., 2018; Schoenberg et al., 2019). In particular, intact male monkeys have shown delayed learning rates compared to intact females with levels of T impacting this sex difference as higher T levels correlated with poorer learning scores (Hagger and Bachevalier, 1991), and habit drinking of alcohol has been observed in males with low T levels (Fukuhara et al., 2017). These effects of T on slowing learning and impairing habit in males provide one possible explanation for why delayed habit is observed in intact males compared to females, and why we detected early habit in these experiments upon the removal of T by castration.

The influences of T on behavior may be caused by its ability to significantly increase dopamine transporter (DAT) binding density in the striatum (Mitchell and Stewart, 1989) as a compensatory result of greater DA release into the intercellular space (de Souza et al., 2009), thus enhancing activity of the DA system in adult male rats. Additionally, significantly more DA neurons have been identified in the male substantia nigra pars compacta (SNc; projects to the striatum supplying it with DA) than in the female SNc (McArthur et al., 2007a,b), which may be attributable to the fact that the male brain is constantly exposed to high levels of E2 and therefore, high concentrations of DA.

Because of the plethora of literature conducted on the effects of estrogens on DA release and habit learning in females (Yoest et al., 2014; Almey et al., 2015; Hussain et al., 2016; Yoest et al., 2018), we were looking specifically at the effects of E2 on habit development in male rats (Experiment 2). Although extensive research has not been conducted on the role E2 plays in the male brain, in 1999, Lammers et al. found that E2 downregulated D2 receptor mRNA in the striatum. Lammers et al., (1999) had speculated that this might be due to E2's ability to increase presynaptic dopamine release (Di Paolo et al., 1985; Disshon et al., 1998) which supports our hypothesis because intact males may be expressing habitual behavior later in instrumental training than intact females due to greater than optimal DA release in the striatum caused by constant high E2 aromatized from T. Based on the preliminary results of Experiment 2 described in Chapter 3 which indicated that E2 replacement in CAST males trained to 160 R-Os produced goal-directed behavior, we do not have sufficient data to conclude that E2 is responsible for the delayed habit seen in intact males.

More recently, as the sex differences caused by gonadal hormones cannot be ignored, the effects of estrogens on the male brain, DA release, and behavior are starting to be studied. In particular, E2 has been found to protect males from vulnerability to addiction of drugs of abuse but enhances motivation for drug taking in female rats (Quigley et al., 2021). These impacts of E2 on motivated behavior and drug seeking were shown to be via activation of G protein-coupled estradiol receptor 1 in the dorsolateral striatum (Quigley et al., 2021), and highlight the often-opposing effects of E2 on male and female behavior. In the future, more extensive research will need to be conducted to determine exactly how E2 is influencing habit development in male rats.

Even though we were specifically looking into the effects of E2 from T on habit development in male rats, T is also consistently converted into DHT, a much more potent androgen. Testosterone and DHT change gene expression by binding directly to androgen receptors (ARs) or, following aromatization to estradiol it can then bind to estrogen receptors (ERs). Both estrogens and androgens employ their effects in one of two mechanisms in the male and female brain: either by binding to membrane receptors to directly effect neurotransmission, or by binding to nuclear receptors which influence gene expression and have indirect effects.

In addition to ERs, ARs have been found to be located on the surface of dopamine neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) – both of which subsequently influence the dorsal striatum – allowing androgen binding to modulate dopamine production and metabolism (Li et al., 2017). Since both estrogens and androgens have receptors which can impact DA release in the striatum, they may both be

playing a critical role in delaying habit development in intact male rats compared to intact females. Since T can be converted to DHT and E2, any of these hormones may be activating receptors and we should consider each of their functions in regulating habit development in male rats.

The effects of DHT on the striatum, motor outputs, and habit are less clearly understood, but one study has implicated DHT and not E2 in causing reduced learning rates – and consequently delaying habit – in intact males (Hagger and Bachevalier, 1991). It was determined that the reversal in sex differences observed in habit formation of visual discrimination was specifically due to the effects of DHT on learning since T replacement alone in OVX females failed to slow the rate of learning comparable to that of intact males (Hagger and Bachevalier, 1991).

Interestingly, gene expression and protein levels of DAT, were increased in the substantia nigra of CAST adolescent males by androgens but not estradiol in adolescent male rats given either T, DHT, or E2 replacement (Purves-Tyson et al., 2014), indicating that AR, not ER, activation is critical for these responses. These findings are contrary to our expectations about the influences of estrogens on habit development in male rats, but support the beliefs that gonadal steroid hormones have opposite effects in males and females and accentuate the need for future studies on the effects of DHT on habit. Thus, if E2 is not found to be the cause of delayed habit development in intact males compared to intact females in future studies, DHT replacement in CAST males could be studied as a potential mechanism regulating habit formation in intact males.

Another important factor that is worth mentioning that may have influenced our results was the timing and effects that castration may have had on our animals. In the present studies, castration of the adult animals used happened no more than seven days prior to their arrival in our colony room. Although these rats were already in adulthood, castration in adolescent rodents has been found to cause changes in gonadal steroid synthesis, medium spiny neuron (MSN) spine density, and DA release. One study on CAST males revealed that castration caused an increase in dopamine neurons in the VTA and SNc which were reversed by DHT and testosterone replacement (Johnson et al., 2010).

Furthermore, testosterone removal by castration increased dopamine turnover – which is often used as a measure of dopamine activity – in the striatum and this stimulatory effect was attenuated by testosterone replacement (Purves-Tyson et al., 2014). Additionally, although MSNs do not exhibit sex differences in neuron density in intact males and females (Meitzen et al., 2011), DHT has been demonstrated to decrease MSN spine density via mGluR5 in CAST males, and this is similar to how estrogens mediate changes on MSNs in females (Gross et al., 2018). These effects of castration on dopamine and MSNs in the striatum indicate that T and DHT suppress DA pathways in intact males, potentially balancing the excitatory effects of estrogens on DA that we believe are causing males to maintain goal-directed responding at a level of instrumental training where intact females express habit.

As mentioned previously, E2 in males originates from aromatized T, but can also be synthesized de novo from cholesterol (McCarthy and Konkle, 2005; Rune and Frotscher, 2005; Garcia-Segura, 2008). In the present experiments, even CAST males

without hormone replacement (Experiments 1A-D) had silastic capsule implants filled with cholesterol as the vehicle. It is unclear whether estrogens can be synthesized from cholesterol to equivalent concentrations of E2 aromatized from T in intact males, but it is worth mentioning this possible effect of cholesterol in these studies as we did not analyze plasma concentrations of E2 postmortem.

Overall, sex differences in structural organization and hormonal activation clearly exist in male and female brains that impact motor outputs and the expression of habitual behavior. In these experiments we confirmed that male gonadal steroid hormones regulate instrumental learning by delaying habitual responding in male rats compared to intact females, and we began to analyze how estradiol specifically influences habit development in males. These findings are important in expanding our understanding of how male steroid hormones function in producing behavioral outputs and may be implicated in sex specific treatments for psychopathologies or addiction when habitual responding becomes maladaptive.

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