ANDROGEN MODULATION OF BEHAVIOURAL FLEXIBILITY IN MALE RATS

by

Valerie Lo

B.A., The University of British Columbia, 2020

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ARTS

in

The Faculty of Graduate and Postdoctoral Studies

(Psychology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2022

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

Androgen modulation of behavioural flexibility in male rats

submitted by Valerie Lo in partial fulfillment of the requirements for

the degree of Master of Arts

in Psychology

Examining Committee:

Kiran Soma, Professor, Psychology, UBC
Supervisor

Stan Floresco, Professor, Psychology, UBC
Supervisory Committee Member

Luke Clark, Professor, Psychology, UBC
Supervisory Committee Member

Abstract

Behavioural flexibility, the ability to adapt behaviour in response to environmental changes, is regulated by the mesocorticolimbic system. Strategy set-shifting is one form of behavioural flexibility, where subjects initially learn one rule to receive a reward (e.g., select the lever illuminated by a visual-cue, or the "light rule"), but then must switch to a novel rule (e.g., select the lever in one position, regardless of visual-cue, or the "side rule"). Androgens, such as testosterone (T), are produced locally within the mesocorticolimbic system, and T treatment impairs set-shifting. Decreasing androgens with the androgen synthesis inhibitor abiraterone acetate (ABI) improves performance of male rats on the strategy set-shifting task using the light-side shift. However, the effect of ABI treatment was small. In this study, we assess different set-shifting procedures to make the strategy set-shifting task more difficult and examine whether this may increase the effect size of ABI treatment. In the first study, rats were assigned to one of six different set-shifting tasks, which required them to perform either the light-side shift or side-light shift, with variable numbers of minimum learning trials for the initial discrimination and with or without 20 reminder trials prior to the set-shift. The side-light shift was significantly more difficult than the light-side shift, as indicated by rats making a greater number of errors to criterion, perseverative errors, regressive errors, and never-reinforced errors on the set-shift and requiring a greater number of trials to criterion to complete the task. In the second study, rats were assigned to (1) ABI or vehicle treatment and (2) the light-side or side-light shift. We replicated our previous findings showing that the side-light shift was significantly more difficult. However, we found no effect of ABI on either type of shifts. These data demonstrate one version of the strategy set-shifting task that is more difficult and provide more insight into the potential role of androgens in behavioural flexibility in male rats.

Lay Summary

As Heraclitus once said, the only constant in life is change. When things change, we often must be flexible and change our behaviour accordingly, depending on the information in our environment. Here, we look at the potential role of the sex hormone testosterone on the male rat's ability to flexibly change their behaviour using a behavioural task called the strategy set-shifting task. Here, rats will first learn one rule for sugar pellets (i.e., press lever on left side, "side rule"), and then they will have to shift to a new rule for sugar pellets (i.e., press lever on top of which light is illuminated, "light rule"). By changing various parts of the task, we show that the side-light shift is more difficult than the light-side shift. Further, by blocking the production of testosterone, we show that there is no difference in ability to flexibly change behaviour in male rats.

Preface

I am the primary author of this thesis and I carried out all experiments at the University of British Columbia (Vancouver Campus). This project was co-supervised by Dr. Soma and Dr. Floresco. Dr. Soma, Dr. Floresco, and I identified the research question and designed the experiment. Debra Bercovici, Melody Salehzadeh, Sofia Gray, Desiree Seib, Jordan Hamden, Cecilia Jalabert, and Ryan Tomm taught me the techniques required to perform the experiment. Cameron Kelsey and Esther Choi assisted with animal husbandry and data collection. I was responsible for ordering necessary materials, animal husbandry, data collection, data analysis, and manuscript writing. Dr. Soma, the supervisor author, provided guidance and feedback on manuscript writing and data analysis. Dr. Floresco also provided guidance and feedback on my manuscript and data analysis. All experiments were conducted in accordance with the Canadian Council for Animal Care and were approved by the Animal Care Committee at the University of British Columbia (protocol # A19-0063).

A version of chapter 2 and chapter 3 were virtually presented at two conferences: the 2021 Canadian Neuroscience Meeting and 2021 Society for Neuroscience meeting.

Lo, V. M., Kelsey, C. R., Floresco, S. B., & Soma, K. K. (2021). Androgen modulation of behavioural flexibility in male rats. Abstract. Presented at SfN Neuroscience 2021, Virtual Conference, Poster Presentation.

Lo, V. M., Floresco, S. B., & Soma, K. K. (2021). Effects of androgens on behavioural flexibility in male rats. Abstract. Presented at CAN-ACN 14th Canadian Neuroscience Meeting, Virtual Conference, Poster Presentation.

Table of Contents

List of Tables

List of Figures

Acknowledgements

First and foremost, I would like to thank Kiran Soma for his exceptional mentorship and support over the last 2 years. Kiran has been incredibly supportive while I hit obstacle after obstacle in my projects, guiding me away from panic and anxiety to problem-solving and learning from my mistakes. Not only has he guided me through the research process, Kiran has also been extremely supportive of and caring towards my own personal growth and my life and career goals. Without Kiran's advice and friendship, I would not be the person I am today and for that, I am incredibly grateful.

Next, I would also like to thank Stan Floresco for his guidance and support on my projects. Stan has always been readily available and supportive, especially through all the behavioural training and testing obstacles that I hit over the last 2 years. I would not have been able to finish these projects without his support. I am also thankful for all the BNS/Floresco lab get togethers, for which Stan is always the gracious host, offering delicious food (especially the softball burgers!) and drinks.

I would also like to thank Debra Bercovici and Ryan Tomm, who have been quick to offer their guidance and support for my projects. I also want to thank Esther Choi and Cam Kelsey, my undergrads, who spent countless hours with me in CDM. You guys made my time there so much more fun!

Finally, I would like to thank everyone in the Soma Lab and Floreseco Lab. Everyone has been extremely helpful, whether that be offering their time to train me on various tasks, help me with my work when I was overwhelmed, discuss my projects and data analysis, or practice my presentations. I may be the one receiving the MA degree and graduating, but research really is a team effort. I would not have been able to do it without every one of you!!

Chapter 1: Introduction

Steroid hormones are produced endogenously and travel through the bloodstream of an organism to regulate physiology. There are five classes of steroid hormones: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens, all of which are derived from cholesterol (Figure 1). Some of the enzymes that produce steroids, such as CYP17A1 which synthesizes androgens including testosterone, are expressed in the brain (Emanuelsson et al., 2018). Thus, these steroids that are produced and act within the brain are termed neurosteroids (Baulieu & Robel, 1990). Since the discovery of neurosteroids, such as testosterone and estradiol, studies have mainly focused on its critical role in aggressive and reproductive behaviour. There has been less focus on their role in other behaviours, including executive functions. Therefore, the focus of this thesis is to elucidate the role of neurally-produced androgens in executive functions.

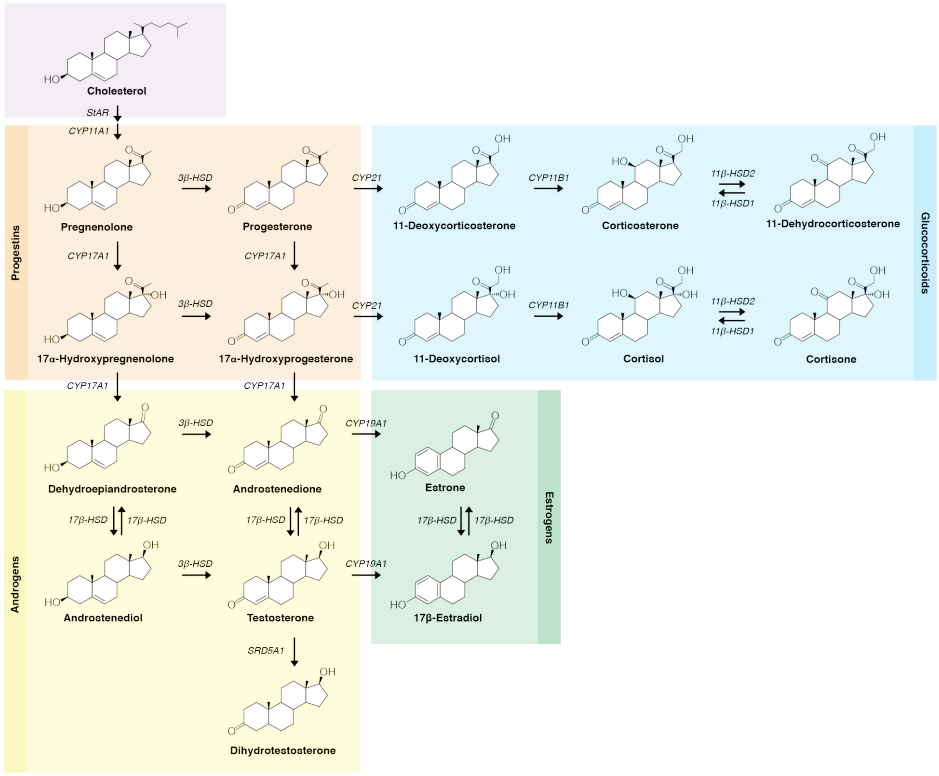

Figure 1: Steroid synthesis pathway. (Salehzadeh, 2019)

Executive function refers to multiple cognitive operations including working memory, inhibitory control, cost/benefit decision-making, risky decision making, and behavioural flexibility, all of which are regulated by the mesocorticolimbic system, which includes the medial prefrontal cortex, nucleus accumbens, and ventral tegmental area (Kesner & Churchwell, 2011). These cognitive functions are vital because they allow organisms to thrive successfully by storing and manipulating information, making thoughtful decisions, and adapting to changes. Interestingly, a few psychiatric and neurological illnesses that present with dysregulation of executive functions, such as autism spectrum disorder, schizophrenia, and depression, also show sex differences in prevalence and symptom presentation, indicating a possible involvement of androgens and estrogens (Abel, Drake & Goldstein, 2010; Werling & Geschwind, 2013; Eid, Gobinath, & Galea, 2019). For example, in schizophrenic patients, lower levels of testosterone are correlated with more severe symptoms (Li et al., 2016). Additionally, there is a positive correlation between fetal testosterone levels and autistic trait, and there is evidence that patients with autism spectrum disorder have higher levels of testosterone and its precursors (Werling & Geschwind, 2013). Furthermore, in males, lower levels of testosterone are correlated with higher levels of anxiety and depressive symptoms, and testosterone treatment improved both anxiety and depressive symptoms (McHenry et al., 2014). Similarly, testosterone treatment also improved symptoms of treatment-resistant depression in females. Thus, investigating the role of neurosteroids in executive functions will provide further insight into the role sex steroids may play in these disorders.

Behavioural flexibility

Behavioural flexibility, the ability to change behaviour to adapt to some environmental or contextual change, is one type of executive function that is impaired in autism spectrum disorder, schizophrenia, and depression (Peters-Scheffer et al., 2013; Waltz, 2017; Uddin, 2021). Behavioural flexibility involves inhibiting previously advantageous behaviours that are now no longer advantageous and adopting a new behaviour. In a world that is constantly changing such as our own, it is important to be able to exhibit behavioural flexibility. This trait provides advantages in the natural environment, such as helping animals like raccoons, skunks, and rodents adapt to life in urban environments (Sol, Lapiedra, & González-Lagos, 2013).

In rodents, behavioural flexibility can be measured by different behavioural paradigms, two of which include reversal learning and set-shifting behaviour. Both require shifting within or between different stimulus dimensions. Stimulus dimensions can be internal to the animal (i.e., egocentric spatial location) or external to the animal (i.e., visual-cue, odour-cue, or sound-cue).

In reversal learning paradigms, animals are required to switch between two strategies belonging to the same stimulus dimension (Floresco, Block, & Tse, 2008). For example, one reversal learning paradigm in operant chambers requires animals to switch between two rules within the egocentric-spatial discrimination for reward (i.e., switch from pressing the left lever to press the right lever).

Behavioural flexibility can also be measured by set-shifting behaviour, wherein "set" refers to the ability to focus solely on one stimulus dimension (Owen et al., 1993). To shift between sets is to shift attention from one stimulus dimension to another dimension (visual-cue to odour-cue). Set-shifting behaviour can be measured through set-shifting paradigms. Whereas reversal learning requires animals to switch between two strategies of the same dimension (right to left),

set-shifting paradigms require subjects to perform a multi-dimensional shift (spatial-left to visual-cue), in response to environmental changes (Floresco et al., 2008). Behavioural flexibility is demonstrated by successful inhibition of previous strategies and learning and maintaining a new strategy. There are various versions of the set-shifting paradigm, such as the maze paradigm, extra/intradimensional shift task (EDS/IDS task), and automated strategy set-shifting paradigm.

In the maze set-shifting paradigm, subjects are placed onto a four-arm plus-shaped maze and are required to switch between two strategies of different dimensions to receive a reward at the end of one arm (Ragozzino, Detrick, & Kesner, 1999; Floresco et al., 2006a, Floresco et al., 2006b). Here, the two dimensions used are visual-cue, which requires animals to respond to visual information, and spatial-response, which requires animals to use egocentric information (Floresco et al., 2006a; Floresco et al., 2006b). Animals are required to learn one discrimination (i.e., visual-cue rule) initially. After completing a certain number of correct consecutive trials, the contingency will change and animals will be required to respond to a novel discrimination (i.e., spatial-response rule) for reward.

The EDS/IDS task requires subjects to dig for reinforcement placed inside one of two bowls (Birrell & Brown, 2000). Here, subjects are required to shift between two dimensions: odour and digging medium. The odour dimension requires subjects to associate an odour with reward, such that they will dig in the bowl with that odour to obtain the reward (i.e., cinnamon). Once animals successfully complete a certain number of consecutive trials, the contingency will change and subjects will be required to respond to digging medium for reinforcement (i.e., paper).

Floresco, Block, & Tse (2008) developed an automated version of the set-shifting paradigm, called the strategy set-shifting paradigm (Figure 2). Conducted within operant chambers, this

task uses levers on the left and right side with a stimulus light situated above each lever. Here, subjects are required to shift between rules of two dimensions (visual-cue and spatial-response) to receive food reinforcement. The visual-cue dimension requires subjects to respond to the stimulus light above each lever such that if the left light is on, the correct response is the left lever, or "light rule". In contrast to this, the spatial-response dimension entails animals to respond to the lever on one side, requiring the use of egocentric spatial information, or "side rule". In the light-side strategy set-shifting paradigm, animals had to shift from using the light rule to using the side rule. In the side-light strategy set-shifting paradigm, animals had to shift from using the side rule to using the light rule.

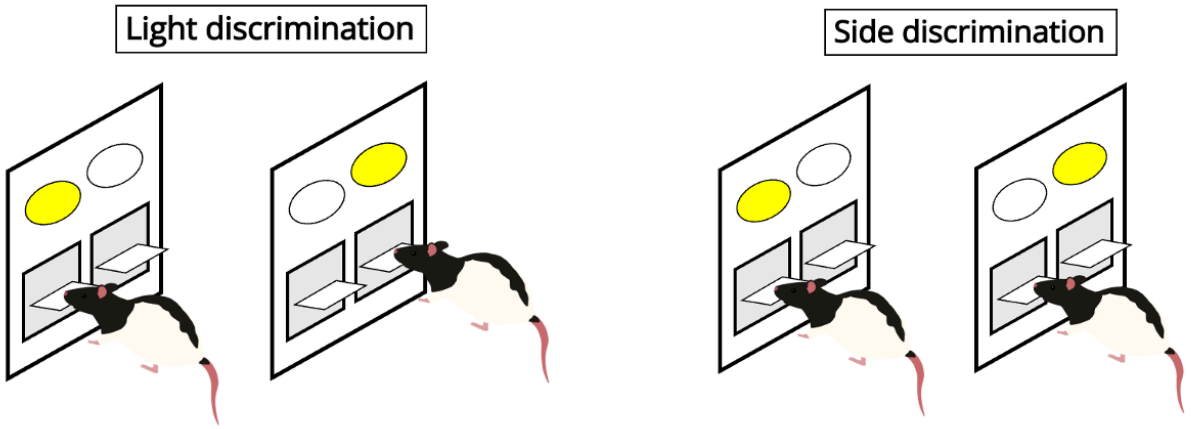

Figure 2: Strategy set-shifting task in an operant chamber. (Adapted from Floresco et al., 2008)

Brain regions implicated in behavioural flexibility

Several regions of the brain are implicated in set-shifting, most notably regions of the mesocorticolimbic system. The mesocorticolimbic system includes the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and dopamine inputs from the ventral tegmental area (VTA) (Tobiansky et al., 2018b). The mPFC consists of three regions, the prelimbic cortex (mPFC-PL), infralimbic cortex (mPFC-IL), and anterior cingulate cortex (Chen, 2017). Of these regions,

mPFC-PL has been implicated in set-shifting. In rodents, mPFC-PL activity increases when switching between rules on the strategy set-shifting paradigm (Del Arco et al., 2017). Additionally, neural activity recordings suggest that mPFC-PL neurons are likely involved in the anticipation of an impending strategy shift (Rich & Shapiro, 2009), and may be involved in detecting rule violations and changes by maintaining a representation of the current rule (Durstewitz, Vittoz, Floresco, & Seamans, 2010). Furthermore, pharmacological inactivation of the mPFC-PL impairs performance on the maze, digging, and strategy set-shifting paradigms (Rich & Shapiro, 2007; Ragozzino et al., 2003; Floresco et al., 2008), suggesting that this area is important for optimal switching between rules of different dimensions.

Other regions of the mesocorticolimbic system, such as the NAc, have also been implicated in set-shifting. According to Floresco et al., (2006a), the subregions of NAc, the NAc core and NAc shell, have differential roles in the maze set-shifting paradigm, where subjects were required to shift between a spatial-response (egocentric) and visual-cue discrimination strategy. Inactivation of the NAc core using a GABA agonist impaired shifting from one strategy to another, while inactivation of the NAc shell improved set-shifting performance. This suggests that the NAc core and NAc shell activity may facilitate and inhibit behavioural flexibility in a maze-based task, respectively.

Dopamine is also used within the mesocorticolimbic system and is involved in reward- and motivation-based learning and behaviours (Bromberg-Martin, Matsumoto, & Hikosaka, 2010). Dopamine, which is produced by dopaminergic neurons in the VTA, is trafficked from the VTA to mPFC-IL, mPFC-PL and NAc core and shell. (Cooper, Robison, Mazei-Robison, 2017). Dopamine signaling within these regions occur via dopamine receptors D1, D2, and D4 in the

mPFC to facilitate behavioural flexibility (Ragozzino et al., 2002; Floresco et al., 2006b), and D1 and D2 in the NAc core and shell (Haluk & Floresco, 2009).

In the mPFC, set-shifting depends on the differential actions of dopamine receptors D1, D2, and D4. In two separate studies, behavioural flexibility in male Long Evans rats were assessed using the maze-based set-shifting paradigm with a shift from response to visual-cue discrimination and vice versa. Infusions of D1 antagonist into the mPFC-IL and mPFC-PL and D2 antagonist and D4 agonist separately into the mPFC impair set shifting performance by selectively increasing perseverative errors (Ragozzino et al., 2002; Floresco et al., 2006b), suggesting that D1 and D2, and not D4, activity is required for optimal behavioural flexibility. Furthermore, infusions of D4 antagonist into the PFC decreased perseverative errors, which is in line with evidence above suggesting that D4 activity is not required for optimal behavioural flexibility. Evidence here demonstrates the differential role of dopamine receptor subtypes in behavioural flexibility, suggesting that the behaviour may depend on the collaboration between the three receptor subtypes.

Additionally, dopamine activity facilitated by D1 and D2 receptors in the NAc are also involved in set-shifting. Haluk & Floresco (2009) demonstrated the differential role of D1 and D2 in behavioural flexibility. Bilateral infusions of D1 but not D2 receptor antagonist into the NAc impaired set-shifting performance on the strategy set-shifting task requiring rats to shift from light-side discrimination. This indicates that in the NAc, D1 but not D2 receptor activity is required for optimal set-shifting.

Finally, the dorsomedial striatum (DMS), which receives cortical input from prefrontal neurons (Terra et al., 2020), has also been implicated in set-shifting. Using the maze-based set-shifting task, Ragozzino et al., (2002) demonstrated that inactivation of the DMS impaired

shifting between a visual-cue rule and response (egocentric) rule. While these animals were able to inhibit the previously advantageous strategy, they were unable to maintain the new strategy, suggesting that the DMS

Sex steroid production

Sex steroids can be produced in several sites, including the gonads and the brain. Until recently, sex steroids were thought to be produced only gonadally via hypothalamic-pituitary-gonadal (HPG) axis signaling (Ruiz-Cortéz, 2012). Here, the sex steroid production pathway begins with the hypothalamus, which releases gonado-tropin releasing hormone to signal the anterior pituitary gland to release into the bloodstream follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LH and FSH then travel systemically to the gonads, signaling them to produce and release sex-specific steroid hormones, such as testosterone, progesterone, and estradiol. Once in the bloodstream, the sex steroids will be transported to different tissues of the body, including the central nervous system, to exert their effects.

More recently, steroid production has been expanded to include locally produced neurosteroids. While gonadally produced sex–steroids can cross the blood-brain barrier and exert effects directly on the central nervous system, the notion that neurosteroids can also be produced *de novo* in various brain regions is now widely accepted (Banks, 2012, Kazuyoshi et al., 1999). There is evidence showing that the brain contains all the materials and enzymes needed to synthesize steroids *de novo*. All steroids are derived from cholesterol, which is found abundantly in the brain (Orth & Bellosta, 2012). The rate–determining step of steroidogenesis depends on the activity of enzyme CYP11A1, which converts cholesterol to pregnenolone (Miller & Auchus, 2011). CYP11A1 mRNA and protein have been detected in the brain (Goascogne et al., 1987;

Stromstedt & Waterman, 1995). Furthermore, the mRNA of other steroidogenic enzymes CYP17A1, CYP21, CYP11B1, and CYP11B2 and the enzyme proteins 17β-HSD and 3βHSD were also found in the brain (Stromstedt & Waterman, 1995; Pelletier, Luu-The, & Labrie, 1995; Emanuelsson et al., 2018; see Mensah-Nyagan et al., 1999 for a review). Taken together, the brain contains all materials necessary for local steroidogenesis. In fact, it is widely accepted that the hippocampus and hypothalamus synthesize testosterone and estradiol locally, where the hormones contribute to learning and memory (see Fester & Rune, 2021 for a review)

Role of neurosteroids in the mesocorticolimbic system

In addition to testosterone being produced in the hippocampus, there are several lines of evidence suggesting that testosterone acts within the mesocorticolimbic system. First, Tobiansky et al (2018a) demonstrated the presence of androgen receptor (AR) mRNA in the VTA, NAc, and mPFC, suggesting that ARs are synthesized locally in the brain. Next, Kritzer (2004) demonstrated that the rat cerebrum contains ARs, while Low, Ma, & Soma (2017) found that many mPFC neurons contain ARs, onto which testosterone binds to alter cell activity, suggesting that mPFC neurons are androgen sensitive. Finally, testosterone has been detected in the brain of gonadectomized animals (Tobiansky et al., 2018a).

Furthermore, testosterone may also be produced locally within the mesocorticolimbic system. Real-time quantitative polymerase chain reaction (RT-qPCR) data showed that mRNA of the enzyme CYP17A1, which is required for androgen synthesis, is expressed in the mPFC, VTA, and NAc (Tobiansky et al., 2018a), its presence suggesting that its products, androgens including testosterone, may be synthesized in those regions. Additionally, the authors also showed that gonadectomized male rats had testosterone in the mPFC, VTA, and NAc, but not

blood, six weeks after their systemic source of testosterone, the testes, were removed. Also, intact male rats who had their testes had higher levels of testosterone in the mPFC, VTA, and NAc when compared to blood, which would not be expected if the only source of testosterone was from the testes. All these findings suggest that testosterone is produced locally within the mesocorticolimbic system and that the mesocorticolimbic system is androgen-sensitive.

As mentioned above, dopamine is used in the mesocorticolimbic system as a signaling molecule. Interestingly, neurosteroids can modulate dopamine levels and signaling. Triemstra, Sato, & Wood (2008) investigated the effects of intracerebroventricular micro-infusions of testosterone on extracellular dopamine levels in the brain. They found that in male Syrian hamsters, high levels (2μg/infusion) but not low levels (1μL/infusion) of testosterone decreased extracellular levels of dopamine in the nucleus accumbens, when compared to control animals. Similarly, Aubele, & Kritzer (2011) demonstrated the effects of gonadectomy and testosterone replacement on dopamine signaling in the prefrontal cortex (PFC). Four days after surgery, gonadectomized animals had lower levels of extracellular dopamine in the PFC compared to control animals. However, 28 days after surgery, gonadectomized animals had greater levels of extracellular dopamine in the PFC than control rats. Both effects were rescued in gonadectomized animals given testosterone replacement. Furthermore, the same group found that projection neurons from PFC to VTA have high levels of ARs, suggesting a potential mechanism through which androgens including testosterone may modulate mesocorticolimbic dopamine system (Aubele & Kritzer, 2012). Taken together, these findings implicate a role of testosterone in dopamine signaling in the brain.

Role of neurosteroids in behavioural flexibility

Testosterone, which acts within the mesocorticolimbic system and may be produced locally within the system, plays a role in behavioural flexibility. In rodents, systemic injections of testosterone impaired performance on multiple versions of the strategy set-shifting paradigm (Wallin & Wood, 2015). Similarly, Rogers (1974) showed that treating male chickens with systemic testosterone impaired behavioural flexibility by selectively increasing perseveration. Decreasing testosterone systemically using cyproterone acetate (an antiandrogen) decreased perseveration and thus improved behavioural flexibility. Likewise, Tomm et al., (2022) showed that systemic administration of CYP17A1 inhibitor abiraterone in intact and gonadectomized rats, which decreases testosterone levels systemically and locally in the brain, improved performance on the strategy set-shifting paradigm by decreasing total errors. The effect size of abiraterone treatment, however, was relatively small.

Systemic treatments such as those above, however, do not allow us to differentiate between the role of testosterone produced peripherally that enters the brain via passive diffusion from testosterone that is produced locally within the brain. It is currently unknown whether testosterone that is locally produced in the mesocorticolimbic system affects behavioural flexibility. This led to the current study, which aims to address this gap in knowledge by determining the role of neurally-produced androgens in strategy set-shifting, an area that has received relatively little attention. To do this, we conducted two separate experiments to (1) increase the sensitivity of the strategy set-shifting paradigm by making it more difficult and (2) determine whether the new paradigm is sensitive to the effects of testosterone.

Chapter 2: Increasing the Difficulty of the Strategy Set-Shifting Paradigm

Introduction

Behavioural flexibility allows an organism to adapt to and succeed in a changing environment (Del Arco et al., 2017). In rodents, one form of behavioural flexibility is set-shifting behaviour, which is measured through different types of set-shifting paradigms (Ragozzino, Detrick, & Kesner, 1999; Birrell & Brown, 2000; Floresco, Block, & Tse, 2008). Here, we used the strategy set-shifting paradigm, developed by Floresco, Block, & Tse (2008), which requires rats to press one of two levers for reward. Located above each lever is a stimulus light (cue-light). To receive reward, rats must shift between the visual-cue dimension (light discrimination), which requires subjects to respond to the cue-light above each lever, and the spatial-response dimension (side discrimination), which requires subjects to ignore the cue-light and respond to the lever on one side. Set-shifting behaviour is mediated by regions of the mesocorticolimbic system, including the prelimbic (PL) region of the medial prefrontal cortex, nucleus accumbens (NAc), and ventral tegmental area (VTA), and the dorsomedial striatum (DMS).

The mesocorticolimbic system is androgen sensitive, as indicated by the presence of androgen receptors (AR) and AR mRNA in the mPFC-PL, mPFC-IL, NAc core, NAc shell, and VTA (Low, Ma, & Soma, 2017; Low et al., 2020; Tobiansky et al., 2018a). Furthermore, regions of the mesocorticolimbic system may synthesize androgens locally. mRNA of CYP17A1, an enzyme critical for androgen synthesis, and CYP19A1 (aromatase), an enzyme involved in converting testosterone to estradiol, have been detected in the mPFC, VTA, and NAc (Tobiansky et al., 2018a). Hsd3b1 (3β-HSD type I) mRNA, involved in multiple steps of the steroidogenesis pathway, was also expressed in VTA. These findings indicate that the mesocorticolimbic system is androgen sensitive and can synthesize androgens *de novo*.

The presence of mRNA of enzymes cannot definitively show that enzyme activity is occurring. However, there are converging lines of evidence that strongly suggest this. Through gold-standard liquid chromatography tandem mass spectrometry, Tobiansky et al., (2018a) showed that gonadectomized male rats had testosterone in the mPFC, VTA, and NAc six weeks after their systemic source of testosterone, the testes, were removed. Similarly, intact male rats who had their testes had higher levels of testosterone in the mPFC, VTA, and NAc compared to blood, which would not be expected if the only source of testosterone was that which passively diffused into the brain from the testes. These two findings suggest that testosterone is in fact produced locally within the mesocorticolimbic system.

If testosterone is produced locally in the brain, it must have a function. There is evidence that testosterone plays a role in behavioural flexibility. In rodents, systemic injections of testosterone impaired performance on multiple versions of the set-shifting paradigm when compared to control animals (Wallin & Wood, 2015). Similarly, Rogers (1974) showed that treating male chickens with systemic testosterone impaired behavioural flexibility by selectively increasing perseveration, while decreasing testosterone using cyproterone acetate (an antiandrogen) decreased perseveration and thus improved behavioural flexibility. Likewise, our group showed that systemic administration of CYP17A1 inhibitor abiraterone acetate, which prevents testosterone synthesis, improved performance on the strategy set-shifting paradigm by decreasing total errors (Tomm et al., 2022). The effect size of abiraterone acetate treatment, however, was relatively small.

Systemic treatments do not allow us to differentiate between the role of testosterone produced peripherally that enters the brain via passive diffusion from testosterone that is

produced locally within the brain. It is currently unknown whether testosterone that is locally produced in the mesocorticolimbic system affects behavioural flexibility.

Elucidating the effects of neurally-produced testosterone on behavioural flexibility requires a paradigm that is more sensitive to the behaviour, as eliminating *only* locally-produced testosterone will likely have a smaller effect than eliminating systemic testosterone. The effect of abiraterone treatment was relatively small in the study by Tomm et al., (2022), leading to concerns of a floor effect when trying to detect a smaller effect, such as that produced by neurally-synthesized testosterone compared by systemic testosterone. Thus, our first goal was to increase the sensitivity of the strategy set-shifting task by making it more difficult. If the task is more difficult, there will be more room to detect improvements in set-shifting behaviour.

The original set-shifting paradigm Tomm et al., (2022) used required animals to shift from the light discrimination (respond to the cue-light above each lever) to the side discrimination (respond to the lever on one side, regardless of location of illuminated cue-light) (light-side shift). There is recent evidence that suggests the opposite shift, the side-light shift, is more difficult. When animals were tested on multiple versions of the strategy set-shifting paradigm, they appeared to have a more difficult time shifting from side-light discrimination compared to light-side discrimination (Walling & Wood, 2015). Likewise, Bercovici et al., (unpublished) demonstrated this same trend. Neither of these studies, however, directly compared the animal's performance on their respective shifts, and they also did not look at whether other components of the task can affect its difficulty. The purpose of this first study was to manipulate the strategy set-shifting task to make it more difficult.

In addition to reversing the order of the shift, we also manipulated the number of learning trials during the initial discrimination, and the presence of reminder trials of the initial

discrimination during the set shift. Our hypothesis was that (1) increasing the number of minimum learning trials for initial discrimination, (2) shifting from side-light discrimination compared to shifting from light-side discrimination, and (3) the presence of reminder trials of the initial discrimination preceding the set-shift would make the task more difficult. We predicted that all three manipulations would increase the number of total errors, perseverative errors, regressive errors, never-reinforced errors, and trials to criterion, indicating the manipulated versions are more difficult than the original version.

Methods

Subjects

Sixty-four adult male Long Evans rats aged 55-79 days, weighing 176-350g from Charles River (Kingston, New York) were used as subjects for this study. Animals were housed in the Centre for Disease Modeling. Animals were pair-housed (2 per cage) in clear polycarbonate cages (48.26cm D x 26.67cm W x 22.35cm H) with stainless steel lids, one PVC pipe, paper towels for nesting, and Nepco BetaChip for bedding. Animals were given food (PicoLab® Rodent Diet 20 EXT, 5R53) *ad libitum* until they weighed 375-425g, which occurred approximately 1.5-9 weeks after arrival. Rats were randomly assigned to one of six conditions, grouped into two types of strategy set shifting tasks, the light-side group and side-light group. Prior to behavioural training, animals were food restricted to 90% of free feeding weight.

Apparatus

Operant chambers (30.5cm x 24cm x 21cm; Med-Associates, St Albans, VT, USA) were used for training and testing. Each chamber was confined within a sound-proof box and

consisted of a fan for ventilation. On the front wall, there were two retractable levers on either side of the food bowl and a circular light above each lever. Reinforcement sugar pellets (45mg; Bioserv) were delivered into the food bowl by a pellet dispenser. The two lights above each lever (cue lights) were used as the stimulus for the visual-cue discrimination. In the front left corner of the sound-proof box containing the operant chamber, there was a 100mA house light that illuminated the chamber. An IBM personal computer using MED-PC was used to collect data.

Behavioural training

Procedures were adapted from Floresco, Block, & Tse (2008). All testing was done at the beginning of the light cycle phase. Training in the operant chambers began one week after food restriction began. Around 20 sugar pellets were given to each rat the day before training began to familiarize the rat with the food reinforcement. On the first day, rats were acclimated to the chambers for 30 minutes, during which food reinforcement was dispensed intermittently. Over the next two days, rats were trained to press the levers using the fixed-ratio 1 program, which required them to meet a criterion of 60 presses within 30 minutes on each lever (left and right), one at a time.

Next, animals began training on pressing retractable levers. Here, levers extended into the operant chamber for 10 seconds, during which rats were required to press the lever to receive reinforcement. There were 90 trials in total, with one trial occurring every 20 seconds. Before each trial began, the chambers were dark, and levers were retracted. At the start of each trial, the house light lit up and 3 seconds later, one lever was extended into the chamber for 10 seconds. If rats responded correctly, by pressing the lever within 10 seconds, they were rewarded one sugar pellet. The levers retracted immediately, and the house light continued to be lit for 4 seconds. If

rats did not respond correctly within 10 seconds, the levers retracted and the house light turned off, no pellet was delivered, and the trial was scored as an omission. Left and right levers were inserted pseudo randomly such that every two trials consisted of one left and one right lever insertion. During this phase of training, the cue lights above each lever were never illuminated. All rats were required to omit less than 10 trials in one session (approximately 5 days of training).

Then, the side bias of each rat was determined. Here, the house light was illuminated, and both levers were extended during any given trial. On the first trial, one pellet was rewarded upon pressing either lever. When the levers were inserted again, rats were required to press the opposite lever to receive a sugar pellet. If rats responded correctly, a sugar pellet was delivered, and the trial ended. If rats responded incorrectly by pressing the lever that they initially pressed, no sugar pellet was delivered, the levers retracted, and the house light turned off. This continued until the rats responded correctly and the trial did not end until rats responded to both levers. Whichever lever the rats responded to more often during the session was determined to be its side bias. If the rats responded equally to both levers, then the rat's initial choice on the first trial was its side bias. Following the determination of side biases, rats were trained on one of two rules, the light discrimination and the side discrimination, depending on which task they were randomly assigned to.

Strategy set-shifting: light-side tasks

There were four different task manipulations used in the light-side tasks, three of which varied the amount of training rats received on the initial light discrimination (N=30 across four tasks). The first task (n=10) (minimum 30) required rats to complete a minimum of 30 trials,

such that the program would continue even if they reached criterion in less than 30 trials after which it would end. Otherwise, trials continued until a rat achieved criterion performance. The second task (n=7) (minimum 100) required rats to complete minimum of 100 trials, with all other parameters being the same as the first task. The third task (n=8) (minimum 2-day) extended the light discrimination training to two days, such that rats were required to complete a minimum of 100 trials over two days (50 trials minimum each day). Even if rats met criterion on the first day, they would still be trained for a second day on the visual-cue discrimination. The three tasks listed here all had reminder trials of the initial discrimination on the set-shift day of the task. The fourth task (n=5) (no reminder) required rats to complete a minimum of 30 trials (identical to the first task), but on the set-shift, they did not receive reminder trials, as described below.

Light discrimination

On the first test day, rats in the light-side group learned the light discrimination. This day consisted of 30-150 trials, with a trial occurring every 20 seconds. At the beginning of each trial, one cue light above the levers was illuminated. 3 seconds later, the house light was illuminated and both levers were extended into the chamber. If the rats responded to the lever above which the light is illuminated, the levers retracted, the house light stayed on for another 4 seconds, and one sugar pellet was delivered. If the rats responded to the opposite lever without the light illuminated, the levers retracted, the house light turned off, and no pellet was delivered. If the rat did not respond within 10 seconds of lever insertion, the levers retracted, the house light turned off, no pellet was delivered, and the response was recorded as an omission. Both cue lights were illuminated once every two trials, with the order randomized. All rats were required to reach a criterion performance of 10 consecutive correct responses, excluding omission.

Shift to side discrimination

For three tasks in this group of tasks (minimum 30, minimum 100, minimum 2-day), the set-shift day started with 20 reminder trials of the light discrimination. After the 20 reminder trials, the correct rule shifted to the side discrimination. The fourth task (no reminder) had no reminder trials. Here, rats were required to ignore the location of the cue lights illuminated and use a spatial-response strategy instead (i.e., always respond to the left lever, regardless of which cue light is illuminated). The correct response was the lever opposite to each rat's side bias, which was previously determined. Criterion was 10 consecutive correct responses and animals responded to 160-180 trials, depending on whether they had 20 reminder trials or not. For all groups, trials continued to 160 or 180 trials, but the program recorded the number of trials and errors when rats first achieved criterion performance.

Strategy set-shifting: side-light tasks

There were two manipulations to the side-light tasks (N=24 across both groups). Both tasks required animals to respond to a minimum of 100 trials, regardless of whether they met criterion before 100 trials or not. The first task had reminder trials (n=14) on the set shifting day while the second task did not have reminder trials (n=10)

Side discrimination

Rats in the side-light group of tasks were initially trained on the side discrimination. This program consisted of 100-150 trials. All other aspects of the training procedure were identical to the training for the side discrimination for the light-side shift tasks.

Shift to light discrimination

On the second day of the test, rats either had 20 reminder trials of the initial side discrimination prior to the shift, or the correct rule immediately shifted to light discrimination. For this rule, animals were required to respond to the lever on top of which the cue light was illuminated. Here, rats were given a total of 310-360 trials over two days to reach criterion of 10 consecutive correct responses.

Behavioural measures assessed

Errors to criterion, the total number of errors required to reach criterion of 10 consecutive correct responses on the initial discrimination and the set-shift were calculated. Errors on the day of the shift were parsed into three types: perseverative errors, regressive errors, and never-reinforced errors.

Perseverative errors are errors where the rat responds to the previously correct rule rather than the currently correct rule. For the light-side groups, this occurred when rats responded to the lever with the light illuminated above, when the correct response was the opposite lever. In the side-light groups, this would occur when rats, who were trained on the left lever, responded to the left lever when the light was illuminated above the right lever. Perseverative errors reflect an inability to inhibit previously advantageous strategies.

Regressive errors occur when a rat responds to the previously correct rule, despite showing an understanding of the currently correct rule. Regressive errors were recorded whenever an animal responded to the previously correct rules after making 5 or less incorrect responses in a block of 16 trials. Regressive errors indicate that while an animal can understand a novel discrimination, they still tend to regress back to the previously correct strategy.

Never-reinforced errors are errors that occur when rats respond to neither the previously correct rule nor the currently correct rule. In both groups, this would be recorded when the light is illuminated above the correct lever, but the rat responds to the opposite lever, which is its side bias. Never-reinforced errors indicate that while a rat can inhibit previously advantageous strategies, they have not learned the correct strategy yet.

Trials to criterion, the total number of trials to reach criterion of 10 consecutive correct responses on both the initial discrimination and set-shift were calculated. This measure excludes reminder trials and omission trials.

The number of correct reminder trials on the set-shift was also calculated. This measure allows us to determine a rat's ability to remember the initial discrimination. Omissions and latency to respond on both the initial discrimination and set-shift were calculated, allowing us to assess levels of motivation for reward.

Data analysis

The primary dependent variables were errors to criterion in both initial and novel discrimination, and perseverative errors, regressive errors, and never-reinforced errors on the set-shift, because total number of errors and error type is a more sensitive measure of set-shifting performance (Tomm et al., 2022). The secondary dependent variables were trials to criterion, omissions, and latency in both initial and novel discrimination, and the number of correct reminder trials.

To examine the effect of manipulating the amount of minimum initial discrimination training on performance on the set-shifting paradigms, we performed a one-way ANOVA for each dependent variable. To examine the effect of reminder trials and type of task, we used a

two-way ANOVA (Task x Reminder) to analyze each dependent variable to determine performance. A total of 10 animals between the four groups performing the light-side shift were removed from analysis because they did meet the criterion of 10 consecutive correct responses in a row on the initial discrimination, resulting in the sample sizes reported earlier. Two of these animals received minimum 30 trials with reminder trials, three animals received minimum 100 trials, two animals received minimum 2-day training, and three animals received minimum 30 trials without reminder trials.

Results

Light-side shift

Amount of training on initial discrimination had no effect on set-shifting

Initial discrimination

We examined whether there were baseline differences to achieve criterion performance between the three groups given varying amounts of minimum training and received reminder trials during the set-shift. There were no differences in errors to criterion ($F(2,22)=0.664$, $p=.525$) (Table 1), trials to criterion ($F(2,22)=0.899$, $p=.421$) (Table 1), omissions ($F(2,22)=0.000$, $p=1.000$) (Table 1), or latency to respond ($F(2,22)=0.307$, $p=.739$) (Table 1).

Set-shift to novel discrimination

Here we examined whether the amount of minimum training required on the initial discrimination would affect set-shifting performance. There were no differences in number of correct reminder trials between groups ($F(2,22)=0.683$, $p=.515$) (Table 1). A one-way ANOVA revealed a trending, but not statistically significant, difference in errors to criterion between groups ($F(2,22)=3.581$, $p=.098$) (Table 1). There were no group differences in perseverative

errors (F(2,22)=1.971, p=.163) (Table 1), regressive errors (F(2,22)=6.275, p=.854) (Table 1), never-reinforced errors (F(2,22)=64.685, p=.109) (Table 1), trials to criterion (F(2,22)=802.701, p=.405) (Table 1), omissions (F(2,22)=2.501, p=.498) (Table 1), or latency to respond (F(2,22)=0.249, p=.782) (Table 1).

Table 1: Behavioural parameters for minimum training on light-side shift. All data are means (SEM).

	Behavioural task		
	Minimum 30 n=10	**Minimum 100 n=7**	**Minimum 2-day n=8**
Initial discrimination			
Errors to criterion	16.60 (5.72)	12.29 (7.02)	7.88 (3.32)
Trials to criterion	60.30 (14.73)	43.57 (18.24)	34.13 (9.79)
Omissions	1.90 (1.36)	1.86 (1.42)	1.88 (0.95)
Latency to respond	1.38 (0.31)	1.42 (0.25)	1.69 (0.33)
Set-shift discrimination			
Reminder trials	16.50 (0.81)	15.29 (0.94)	16.88 (1.11)
Errors to criterion	30.80 (5.60)	18.86 (3.17)	18.62 (2.95)
Perseverative errors	17.90 (3.32)	11.00 (1.51)	12.38 (2.01)
Regressive errors	7.60 (2.30)	3.43 (1.25)	2.62 (0.82)
Never-reinforced errors	5.30 (2.46)	4.43 (1.94)	3.63 (1.71)
Trials to criterion	76.00 (10.03)	57.86 (10.90)	61.75 (9.15)
Omissions	1.00 (0.47)	1.86 (1.10)	0.75 (0.31)
Latency to respond	1.00 (0.14)	0.87 (0.23)	1.04 (0.15)

p>.05 for all comparisons

Comparing tasks

The side-light shift is more difficult than the light-side shift

Initial discrimination

Here we determined whether there were any differences in learning the initial light or side discrimination, and whether animals with reminder or without reminder trials performed differently at baseline. There was a significant effect of task, but not reminder trials, on errors to

criterion (task: F(1,35)=12.572, p=.001; reminder: F(1.35)=1.321, p=.258; task x reminder: F(1,35)=0.090, p=.765) (Figure 3A). There was also a significant effect of task, but not reminder trials, on trials to criterion (task: F(1,35)=7.583, p=.009; reminder: F(1,35)=1.240, p=.273; task x reminder: F(1,35)=0.658, p=.423) (Figure 3B). Therefore, regardless of presence of reminder trials, the initial light discrimination was easier to learn than the initial side discrimination. There was also a significant effect of task, but not reminder trials, on latency to respond (task: F(1,35)=4.535, p=.040; reminder: F(1,35)=0.005, p=.945; task x reminder: F(1,35)=0.100, p=.754) (Table 2). Neither task nor reminder trials had a significant effect on omissions (task: F(1,35)=0.170, p=.683; reminder: F(1,35)=0.466, p=.500; task x reminder: F(1,35)=1.061, p=.310) (Table 2).

Set-shift to novel discrimination

Here we examined whether the type of shift or the presence of reminder trials would affect performance on the set-shift to a novel discrimination. There were no differences in number of correct reminder trials (F(1,22)=0.556, p=.464) (Table 2). There was a significant effect of task, but not reminder trials, on total number of errors to criterion (task: F(1,35)=18.785, p<.001; reminder: F(1,35)=0.851, p=.363, task x reminder: F(1,35)=0.003, p=.957) (Figure 3C), suggesting that the side-light task was significantly more difficult than the light-side task, regardless of whether reminder trials were present or not. There was also a significant effect of task, but not reminder trials, on regressive errors (task: F(1,35)=10.877, p=.002; reminder: F(1,35)=0.330, p=.570; task x reminder: F(1,35)=0.786, p=.381) (Figure 3E), never-reinforced errors (task: F(1,35)=10.881, p=.002; reminder: F(1,35)=2.559, p=.119; task x reminder: F(1,35)=0.169, p=.684) (Figure 3F), and a trending effect of task, but not reminder trials, on perseverative errors (task: F(1,35)=3.614, p=.066; reminder: F(1,35)=1.732, p=.197;

task x reminder: $F(1,35)=0.981$, $p=.329$) (Figure 3D). Regardless of reminder trials, animals shifting from side-light made significantly more regressive errors and never-reinforced errors, with a trending increase in perseverative errors. Additionally, there was a significant effect of task, but not reminder trials, on trials to criterion (task: $F(1,35)=32.862$, $p<.001$; reminder: $F(1,35)=1.333$, $p=.256$; task x reminder: $F(1,35)=0.146$, $p=.705$) (Table 2). Animals required more trials to criterion to complete the side-light shift than light-side shift.

Next, we investigated whether these results were due to differences in motivation by assessing latency to respond and number of omissions. There was no effect of task or reminder trials on latency to respond (task: $F(1,35)=2.138$, $p=.153$; reminder: $F(1,35)=0.976$, $p=.330$; task x reminder: $F(1,35)=0.075$, $p=.786$) (Table 2). There was a trending effect of task, but not reminder trials, on number of omissions (task: $F(1,35)=3.756$, $p=.061$; reminder: $F(1,35)=.013$, $p=.911$; task x reminder: $F(1,35)=.131$, $p=.720$) (Table 2).

Altogether, these data suggest that the side-light shift is more difficult than the light-side shift, as indicated by an increase in errors to criterion, perseverative errors, regressive errors, never-reinforced errors, and trials to criterion on the set-shift. This effect was not driven by differences in motivation level. Furthermore, when rats are naïve, the side discrimination may be more difficult to learn than the light discrimination.

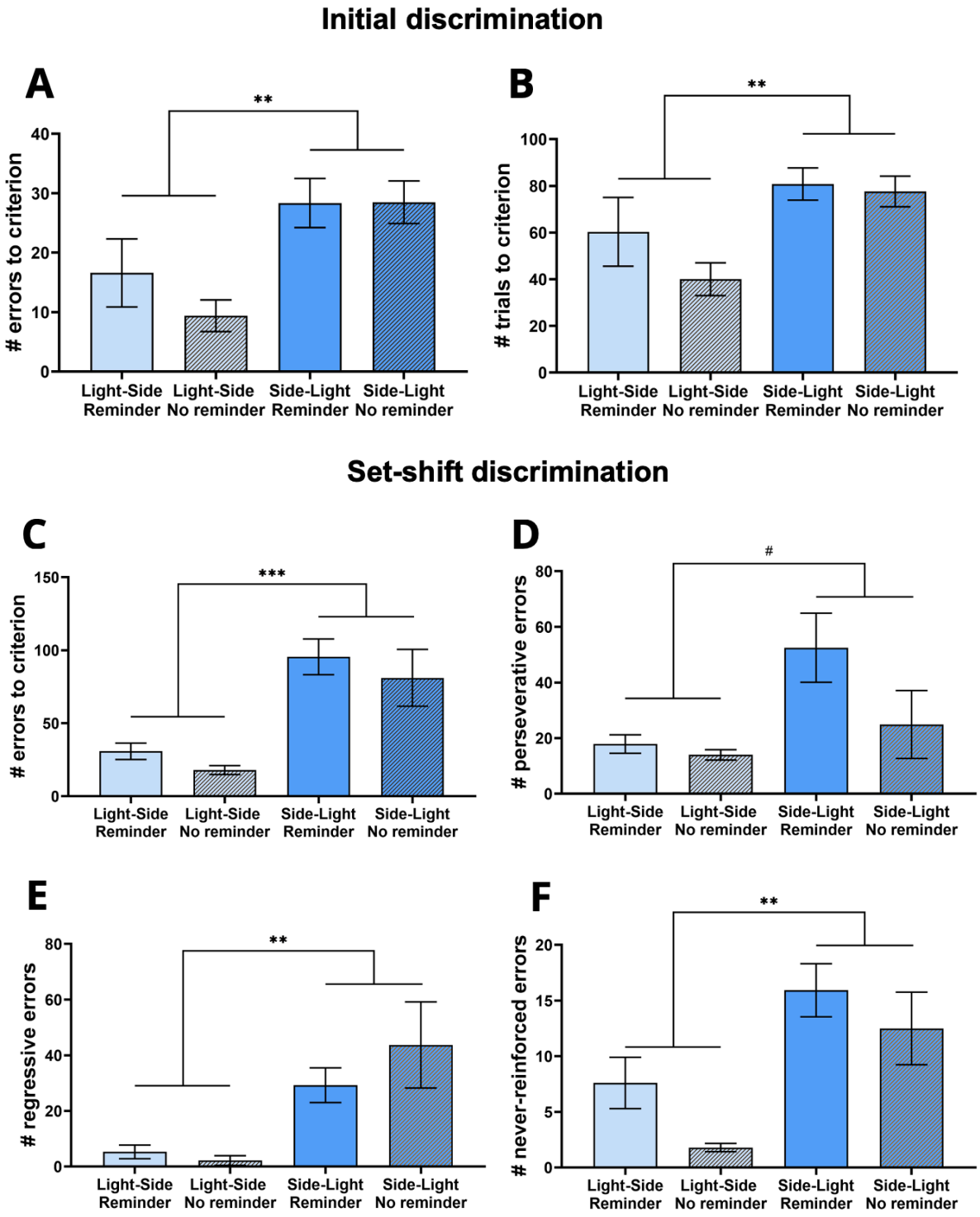

Figure 3: Performance on strategy set-shifting tasks with and without reminder trials. There were four groups: light-side + reminder (n=10), light-side + no reminder (n=5), side-light + reminder (n=14), side-light + no reminder (n=10). **(A-B)** Initial discrimination. **(A)** Errors to criterion. **(B)** Trials to criterion. **(C-F)** Set-shift discrimination. **(C)** Errors to criterion. **(D)** Perseverative errors. **(E)** Regressive errors. **(F)** Never-reinforced errors. Bars represent mean +/- standard error of the mean (SEM). Asterisk (*) indicates significance, * $p < .05$, ** $p < .01$, *** $p < .001$. Number sign (#) indicates trending to significance, # < .10.

Table 2: Selected behavioural parameters for type of tasks and reminder trials. All data are means (SEM).

	Behavioural task			
	Light-side Reminder n=10	**Light-side No reminder n=5**	**Side-light Reminder n=14**	**Side-light No reminder n=10**
Initial discrimination				
Omissions	1.90 (1.36)	0.00 (0.00)	1.21 (0.38)	1.60 (1.38)
Latency to respond	1.08 (0.10)	1.00 (0.18)	1.45 (0.19)	1.50 (0.23)
Set-shift discrimination				
Reminder trials	16.50 (0.81)	-	15.64 (0.78)	-
Trials to criterion*	76.00 (10.03)	53.80 (9.13)	251.64 (21.92)	207.50 (40.40)
Omissions#	1.00 (0.47)	0.20 (0.20)	6.07 (2.29)	7.60 (4.83)
Latency to respond	1.00 (0.13)	0.76 (0.16)	1.23 (0.18)	1.23 (0.18)

* = p<.001 when comparing task type
# = p<.10 when comparing task type

Comparing cohorts of animals

Exploratory analyses were also conducted to determine if there were any differences in performance between the first two cohorts of 16 animals each, which were distributed between the minimum 30, minimum 100, and minimum 2-day groups.

Initial discrimination

Analysis revealed significant differences in errors to criterion ($t(12.057)=2.498$, $p=.028$) (Table 3), and trials to criterion ($t(13.527)=2.596$, $p=.022$) (Table 3) between the two cohorts. There were no differences in omissions ($t(18.214)=-0288$, $p=.388$) (Table 3) or latency to respond ($t(23)=-0.392$, $p=.698$) (Table 3).

Set shift to novel discrimination

There was a trending difference in never-reinforced errors between the two cohorts ($t(11.649)=1.917$, $p=.08$) (Table 3). However, there were no cohort effects on errors to criterion

(t(11.916)=1.559, p=.145) (Table 3), perseverative errors (t(14.208)=0.404, p=.692) (Table 3), regressive errors (t(13.905)=1.342, p=.201) (Table 3), trials to criterion (t(23)=1.143, p=.265) (Table 3), number of correct reminder trials (t(23)=0.281, p=.781) (Table 3), omissions (t(23)=-0.596, p=.557) (Table 3), or latency to respond (t(23)=1.452, p=.160) (Table 3).

Table 3: Behavioural parameters for first two cohorts. All data are means (SEM).

	Cohort	
	1 n=16	**2 n=16**
Initial discrimination		
Errors to criterion	21.27 (5.91)[a]	5.79 (1.90)[b]
Trials to criterion	70.64 (14.83)[c]	28.86 (6.24)[d]
Omissions	1.64 (1.25))	2.07 (0.84)
Latency to respond	1.41 (0.30)	1.55 (0.20)
Set-shift discrimination		
Errors to criterion	28.73 (5.66)	19.50 (1.75)
Perseverative errors	15.00 (3.21)	13.57 (1.48)
Regressive errors	6.45 (2.35)	3.00 (1.04)
Never-reinforced errors	7.27 (2.18)[e]	2.93 (0.62)[f]
Reminder trials	16.45 (0.76)	16.14 (0.76)
Trials to criterion	73.82 (11.41)	60.50 (5.20)
Omissions	0.91 (0.44)	1.36 (0.57)
Latency to respond	1.13 (0.13)	0.86 (0.13)

p<.05 a vs b, c vs d
p<.10 e vs f

Discussion

This current study investigated whether various manipulations of the strategy set-shifting paradigm would make the task more difficult for rodents. We found that the amount of training, and the presence or absence of reminder trials did not influence the difficulty of the task. This is indicated by no differences between groups that received differing amounts of minimum training, and groups that received or did not receive reminder trials, in any behavioural

parameters. We did, however, demonstrate that the side-light shift is more difficult than the light-side shift, regardless of whether reminder trials were present. This is indicated by a significantly greater number of trials to criterion, errors to criterion, regressive errors, and never-reinforced errors, and a trending increase in perseverative errors, when animals performed the side-light shift versus the light-side shift. Additionally, for naïve rats, the initial side discrimination was more difficult to learn than the initial light discrimination, as indicated by a greater number of trials to criterion and errors to criterion when learning the side discrimination. Finally, we found there were differences in initial discrimination learning between the first two cohorts of animals.

Minimum amount of training did not increase difficulty

To increase the difficulty of the task, we manipulated the amount of minimum training required on the initial discrimination, and the presence or absence of reminder trials, anticipating that it would make the shift to a novel rule more difficult. The amount of minimum training on the initial discrimination did not affect performance on the shift. This suggests that increased exposure to the initial discrimination does not affect ability to learn a novel strategy.

Our results contrast with a study by Garner et al., (2006), which found that overtraining on the initial discrimination impaired extradimensional set-shifting behaviour in mice. Here, mice were given a task that required them to dig through bowls of different odour and mediums to find reward. The task involved a circuit of behavioural paradigms, including reversal learning and set-shifting. Prior to this set-shift, mice were either overtrained on the previous strategy or not. Overtrained mice performed significantly worse on the set-shift, requiring a greater number of trials to criterion, and making more errors to criterion than non-overtrained mice.

One main difference between the two studies is our procedure to overtrain. Garner et al., (2006) gave overtrained mice 50 extra trials of training after meeting criterion, which was almost three times the number of trials required to meet criterion. On the other hand, our animals were given a minimum number of trials and/or days of learning, a number that may or may not have exceeded the trials to criterion for any animal. Thus, in our study, only animals who met criterion in fewer trials than the assigned minimum number of trials would be given additional exposure to the initial discrimination. Furthermore, reminder trials offered animals an additional 20 extra trials of exposure to the initial discrimination, which was less than half the number of trials required for both discriminations. Because of these two reasons, not all animals in our study, who were assigned a greater number of minimum trials, extra day of training, or reminder trials can be considered "overtrained animals". Therefore, our animals, on average, may not have had enough overtraining to produce any meaningful difference in set-shifting. To assess the effects of overtraining accurately, future studies will need to ensure each rat receives a standardized amount of training *after* meeting criterion.

For naïve animals, the light discrimination is easier than the side discrimination

Naïve animals learned the light discrimination faster than the side discrimination, as indicated by a decrease in trials to criterion and errors to criterion. One reason for this may be due to the increased salience of the cue-lights. Rats tend to find brighter cue-lights more salient than less bright cue-lights (Farina et al., 2015). The day rats learn the initial discrimination is also the first time the cue-lights are turned on, making the cue-light both novel and bright, and therefore, presumably, more salient than the spatial position of the levers, a dimension that the rats have been exposed to previously through the side preference test. If the cue-lights are more

salient than spatial location, animals may be more inclined to pay attention to and follow the light, resulting in more rapid learning of the light discrimination. Additionally, because the cue-lights are illuminated while animals learn the initial side discrimination, it may also act as a distractor, making the side discrimination more difficult to learn.

Data reported by Floresco, Block, & Tse (2008) are in keeping with this notion. They found that naïve animals required a significantly fewer number of trials to criterion to learn the light discrimination compared to the side discrimination. Floresco et al., suggested this was due to the novel cue-lights being more salient, attracting the animal's attention and causing it to approach the cue-light and press the lever. The cue-light, in this case, would be a novel "object" that rats may be curious about. Furthermore, Floresco et al., also suggest that the light discrimination is preferred because of the rat's preference towards strategies that involve alternation of responses (Dias & Aggleton, 2008). The light discrimination requires rats to switch between responding to left and right lever rather than responding only to one lever on one side.

In a similar vein, Floresco et al., (2006) demonstrated other visual-cue discriminations are also easier to learn in the maze version of the set-shifting paradigm. The visual-cue discrimination here was a black and white striped pattern placed onto the maze. During initial discrimination, animals learned the visual-cue discrimination (~40 trials, ~60 trials) faster than the side discrimination (~65 trials, ~70 trials), respectively. Furthermore, Ragozzino (2002) also found a similar effect, with animals requiring fewer trials (~60 trials) to learn the visual-cue discrimination than the side discrimination (~75 trials). This is similar to our present study, which also demonstrated that the light discrimination, which is part of the visual-cue dimension, is easier to learn than the side discrimination.

In contrast to our findings, however, Wallin & Wood (2015) demonstrated the opposite effect in operant chambers. The light discrimination appeared to be more difficult to learn, requiring ~110 trials to criterion, whereas side discrimination required ~60. Where Wallin & Wood used adolescent male rats, we used adult male rats. Adolescent and adult rats are biased towards different strategies, with adolescence enhancing repetitive response behaviour, of which is involved in the side discrimination. This is certainly possible, but another reason could be because at 5 weeks, a rodent's brain is still undergoing refinement and maturation (Semple et al., 2013), and therefore may not be utilizing the same, or refined, cognitive mechanisms as an adult rodent. Indeed, until postnatal day 50, adolescent rats performed significantly worse compared to adult rats when the task required a greater cognitive load (Kirschmann et al., 2019). Here, the light discrimination may involve a greater cognitive load than the side discrimination, as it requires animals to pay attention to the cue-light. Whereas for the side discrimination, it is sufficient for animals to ignore the cue-light.

Exploratory analyses revealed significant cohort effects in learning the initial light discrimination between the first two cohorts. Our first two cohorts of 16 animals each were distributed between the minimum 30, minimum 100, and minimum 2-day groups. The second cohort performed significantly better on the initial light discrimination, pointing to individual differences that may have led to differences in baseline performance. Some of this variability may contribute to differences in components required to learn the light discrimination, such as vision, ability to ignore irrelevant cues, and ability to remember the previous response. Therefore, we do not know whether our finding that the light discrimination is easier to learn than the side discrimination is being driven by cohort differences that is skewing our data or an actual difference. Further research will be required to tease this apart.

Side-light shift is more difficult than light-side shift

Animals performing the side-light shift performed significantly worse than animals performing the light-side shift on all behavioural parameters, suggesting that the side-light shift is more difficult for rodents. Previous research has yielded inconsistent data on this effect.

Wallin & Wood (2015)'s study shows that the side-light shift is more difficult than the light-side shift. For control animals in the study, the side-light shift required ~250 trials to criterion whereas light-side shift required ~50. Furthermore, Bercovici et al., (unpublished) also demonstrated that side-light shift is more difficult, with animals performing side-light shift requiring ~100 trials to criterion whereas light-side shift required ~65. In this study, however, animals were only given one day to learn the shift rather than multiple days, resulting in some animals who never met criterion on the light discrimination. Both studies support our finding that the side-light shift is more difficult than the light-side shift.

Floresco et al., (2008) demonstrated that the light-side shift was significantly more difficult than side-light shift, as indicated by a greater number of trials to criterion. While we used a criterion of 10 consecutive correct responses, Floresco et al., used a criterion of 8 consecutive correct responses. This means not only did animals receive less training on the initial discrimination, they also would have met criterion faster. Had we also used a criterion of 8 consecutive correct responses, we may have found a similar effect. Furthermore, in our study, the house light was located at the left front corner of the box holding the operant chamber, while Floresco et al., had the house light inside the operant chamber on the wall opposite to the levers and cue-lights. Therefore, in addition to inhibiting the previous side discrimination strategy, animals in our study would also have to learn to discriminate between the two cue-lights and the house light and ignore the house light while paying attention to the cue-light. This discrepancy

may have made the light discrimination more difficult to learn for non-naïve rats, compared to the side discrimination, which does not require any interaction with the house light.

One reason we think the side-light shift may be more difficult is because it may require a greater number of executive functions, and therefore increase the cognitive load. The enhanced difficulty of side-light shift can be attributed to cognitive load. According to Norman and Bobrow (1975), the brain has a finite amount of resources, and completing multiple tasks will require drawing from the shared pool of limited resources. Using more resources for any single task will lead to having less resources available for other tasks. Although this study was about humans, it is reasonable to assume that the rat brain also contains a limited amount of resources for the rat to use at any time. Therefore, a greater amount of cognitive load, such as tasks that require integrating multiple pieces of information, like requires a greater amount of resources.

It can be argued that the side-light shift requires more cognitive resources than the light-side shift. Animals shifting from side-light initially learned that the cue-light is an irrelevant cue and may stop paying attention to it. Because of this, it is reasonable to believe that the cue-light and house light, positioned in the front left corner outside of the operant chambers, may become indistinguishable for the animal. Therefore, when the animal is required to shift to the light discrimination, the animal will have to (1) inhibit the previous side strategy, (2) realize that the cue-lights are relevant now, (3) distinguish between illumination from the cue-lights and the house light, and (4) respond only to the cue-light and not the house light. In contrast to this, animals who initially learned the light discrimination would have already learned to distinguish between the cue-light and house light. Also, both lights are now irrelevant for the side discrimination. Here, animals would be required to (1) inhibit their response to the cue-lights, (2) realize the position of lever, and not cue-lights, is relevant, and (3) respond to the correct side.

Compounding onto the greater amount of cognitive resources required to perform the side-light shift, responding to the light rule may also require a greater amount of movement coordination. For the side discrimination, rodents can theoretically remain in one position in the chamber, press the lever when it extends, move over slightly to obtain the reward, and return to its position to wait for the next lever extension. This discrimination may not require much movement coordination. However, for the light discrimination, rodents are required to pay attention to the cue-light, move to the position of the cue-light, press the lever when it extends, obtain its reward, and then wait for the cue-light to illuminate again to repeat the steps. Compared to the side discrimination, the light discrimination appears to require a greater amount of movement coordination, thus potentially further increasing the cognitive load.

If the side-light shift requires more cognitive resources than the rat possesses, it may have deleterious effects on learning and memory, such that the rat will experience more difficulty learning and remembering the novel strategy. Indeed, Kirschmann et al., (2019) supports this, as they demonstrated that as the cognitive load required for a task increased, successful responding decreased on a working memory task.

In conclusion, we found that the minimum amount of training and the presence or absence of reminder trials did not affect behavioural flexibility on the strategy set-shifting paradigm. Additionally, for naïve rats, the light discrimination is easier to learn than the side discrimination. Finally, the side-light shift is more difficult than the light-side shift.

Chapter 3: Effect of Abiraterone Acetate on Two Set-Shifting Paradigms

Introduction

Steroids are produced in multiple organs including the gonads, adrenal cortex, and the brain. Steroids that are produced in the brain are called neurosteroids (Baulieu & Robel, 1990). Neurosteroids act within the brain to modulate brain activity through inhibitory and excitatory actions via intracellular and membrane-bound receptors (Paul & Purdy, 1992; Wang, 2011) Researchers have investigated the role of neurosteroids in learning and memory processes (Ratner, Kumaresan, & Farb, 2019), aggressive behaviour (Soma et al., 2008), reproductive behaviour (King, 2013), and anxiety, depression, and schizophrenia (Ratner et al., 2019; Zorumski et al., (2013); Cai et al., 2018). There has not been much research, however, on the role of neurosteroids in executive functions. Thus, the goal of this study is to determine the role of neurally produced androgens on executive functions such as behavioural flexibility.

The mesocorticolimbic system, which includes the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and ventral tegmental area (VTA), is involved in higher executive functioning (Tobiansky et al., 2018b), and is androgen sensitive. Androgen receptors (AR) and AR mRNA have been in the prelimbic (mPFC-PL), infralimbic (mPFC-IL), NAc core, NAc shell, and VTA (Low, Ma, & Soma, 2017; Low et al., 2020; Tobiansky et al., 2018a).

The mesocorticolimbic system also contains enzymes required to synthesize androgens and estrogens locally. Our lab detected mRNA of CYP17A1, an enzyme critical for androgen synthesis, and CYP19A1 (aromatase), an enzyme involved in converting testosterone to estradiol, in the mPFC, VTA, and NAc (Tobiansky et al., 2018a). Hsd3b1 (3β-HSD type I) mRNA, involved in multiple steps of the steroidogenesis pathway, was also expressed in VTA. Furthermore, CYP11A1, an enzyme required for the first step of steroidogenesis, conversion of

cholesterol into pregnenolone, has also been detected in the cerebral cortex (Giatta et al., 2019). Finally, cholesterol, which all steroids are derived from, is found abundantly in the brain (Orth & Bellosta, 2012). These findings suggest that the mesocorticolimbic system is capable of synthesizing androgens *de novo*.

While the presence of enzyme mRNA does not definitively demonstrate enzyme activity, there are converging lines of evidence that strongly support this. Through liquid chromatography tandem mass spectrometry, Tobiansky et al., (2018a) showed that gonadectomized male rats had testosterone in the mPFC, VTA, and NAc six weeks after their systemic source of testosterone, the testes, were removed. Similarly, intact male rats with testes had higher levels of testosterone in the mPFC, VTA, and NAc compared to blood, which would not be expected if the only source of neural testosterone was that which passively diffused into the brain from the testes. These findings suggest that testosterone is produced locally within the mesocorticolimbic system.

One of the higher executive functions the mesocorticolimbic system mediates is behavioural flexibility. In rodents, one form of behavioural flexibility is set-shifting behaviour, which is measured through different types of set-shifting paradigms (Ragozzino, Detrick, & Kesner, 1999; Berrell & Brown, 2000; Floresco, Block, & Tse, 2008). The strategy set-shifting paradigm, developed by Floresco et al., requires rats to shift between two rules of different dimensions (light discrimination and side discrimination) for reward. Set-shifting behaviour is measured through the animal's performance on the task.

Testosterone plays a role in behavioural flexibility. In rats, systemic injections of testosterone impaired performance on multiple versions of the set-shifting paradigm (Wallin & Wood, 2015). Similarly, Rogers (1973) showed that treating male chickens with systemic testosterone impaired behavioural flexibility by increasing perseveration, while decreasing

testosterone using cyproterone acetate (an antiandrogen) decreased perseveration and thus improved behavioural flexibility. Likewise, our group showed that systemic administration of CYP17A1 inhibitor abiraterone acetate (ABI), which decreases testosterone levels, improved performance on the strategy set-shifting paradigm by decreasing total errors (Tomm et al., 2022). However, it is currently unknown how locally produced testosterone in the mesocorticolimbic system affects behavioural flexibility, since systemic treatments do not allow us to differentiate between systemic testosterone that passively diffuses into the brain and locally produced testosterone.

Elucidating the effects of locally produced testosterone on set-shifting requires a paradigm that is more sensitive to the behaviour, since eliminating neurally-produced testosterone *only* will likely have a smaller effect than eliminating systemic testosterone. The effect of abiraterone treatment was relatively small in the study by Tomm et al., (2022), which led to concerns of a floor effect when trying to detect a smaller effect, such as that possibly produced by neurally synthesized testosterone. Therefore, in chapter 2, we increased the sensitivity of the strategy set-shifting task by making it more difficult. With a more difficult task, there will presumably be a greater ability to detect improvements in set-shifting behaviour.

In Chapter 2, we demonstrated that the side-light shift is more difficult to perform than the light-side shift, which was used by Tomm et al., (2022). While the side-light shift may be more difficult for rats, it is unclear whether this shift is sensitive to the effects of androgens. Thus, the aim of this experiment was to determine whether systemic elimination of testosterone would impact performance on the modified strategy set-shifting paradigm.

In this experiment, we administered systemic treatment of ABI or vehicle to animals and assessed their performance on the light-side and side-light versions of the strategy set-shifting

paradigm. Our hypothesis was that (1) abiraterone treatment will improve performance on both versions of the task, (2) the modified paradigm (side-light shift) will be more difficult than the original paradigm (light-side shift), and (3) the effect size of ABI will be greater for the side-light shift. We predicted that animals given abiraterone acetate would perform better on both paradigms compared to animals given vehicle, specifically by having a lower number of trials and errors to criterion, perseverative errors, regressive errors, and non-perseverative errors on the set-shift. Additionally, we predict the effect size between ABI and vehicle animals will be greater when performing the side-light shift versus the light-side shift. Finally, we predict that animals performing the side-light shift will have a greater number of trials and errors to criterion, perseverative errors, regressive errors, and never-reinforced errors on the set-shift.

Methods

Subjects

Sixty-four adult male Long Evans rats aged 54-76 days and weighing 210-325g from Charles River (Kingston, New York) were used as subjects for this study. Animals were initially pair-housed (2 per cage) in clear polycarbonate cages (48.26cm D x 26.67cm W x 22.35cm H) with stainless steel lids, one PVC pipe, paper towels for nesting, and Nepco BetaChip for bedding. Animals were given food (PicoLab® Rodent Diet 20 EXT, 5R53) *ad libitum* until they weighed 325-375g, which occurred approximately 1.5-5 weeks after arrival. After reaching this weight, animals were single-housed and 1 week food restriction began, during which animals were food restricted to 90% of free feeding weight. Rats were randomly assigned to one of four groups: light-side shift plus abiraterone acetate (ABI) (n=11), light-side shift plus vehicle (VEH) (n=14), side-light shift plus ABI (n=15), and side-light shift plus VEH (n=15).

Apparatus

Operant chambers (30.5cm x 24cm x 21cm; Med-Associates, St Albans, VT, USA) were used for training and testing. Each chamber was confined within a sound-proof box and consisted of a fan for ventilation. On the front wall, there were two retractable levers on either side of the food bowl and a circular light above each lever. Reinforcement sugar pellets (45mg; Bioserv) were delivered into the food bowl by a pellet dispenser. The cue lights above each lever were used as the stimulus for the visual-cue discrimination task. In the front left corner of the sound-proof box containing the operant chamber, there was a 100mA house light that illuminated the chamber. An IBM personal computer using MED-PC was used to collect data.

Drug treatment

Abiraterone acetate (ABI) (MedChem Express; LOT 28291; stored at -20^{o}C; dose 40mg/kg) or vehicle (VEH) treatment began on the first day of retractable lever training and lasted for the remaining duration of the experiment (a total of 9 days). ABI was delivered via the VEH, which was a 1g food pellet made from 50/50 mixture of peanut butter (Kraft, smooth) and rat chow (PicoLab® Rodent Diet 20 EXT, 5R53). Previously, our lab has shown that 40mg/kg dose of ABI for 4 days is sufficient to eliminate testosterone systemically, and in the brain (Tomm et al., 2022). Additionally, European Medicines Agency (2011) showed that ABI levels peaked in the brain 4 hours after ABI treatment. Therefore, here, our ABI animals were treated with a dose of 40mg/kg of ABI 4 hours prior to behavioural training or testing, while our VEH animals received VEH food pellets 4 hours prior to training or testing.

The average weight of all rats in a cohort were used to calculate the amount of ABI needed for a dose of 40mg/kg per food pellet the day before treatment began. To ensure the ABI food

pellet was 1g, the amount of ABI needed for each food pellet was subtracted from the amount of rat chow needed. ABI and VEH food pellets for the duration of the experiment were created the day before treatment began and the weights of animals were monitored to ensure the average weight stayed consistent.

To prevent contamination, four of the eight operant chamber boxes were assigned to ABI animals and the other four to control animals. Levers were wiped down with 70% ethanol between each animal. Gloves and gowns were changed, and surfaces were wiped down with 70% ethanol prior to handling, weighing, and feeding each group of animals.

Behavioural training

Procedures were adapted from Floresco, Block, & Tse (2008). All testing was done during the light phase. Training in the operant chambers began one week after food restriction. Around 20 sugar pellets were given to each rat the day before training began to familiarize the rat with the food reinforcement. On the first day, rats were acclimated to the chambers for 30 minutes, during which food reinforcement was dispensed intermittently. Over the next two days, rats were trained to press the levers using the fixed-ratio 1 program, which required them to meet a criterion of 60 presses within 30 minutes on each lever (left and right), one at a time.

Then, animals began training on pressing retractable levers. This is also when daily ABI or VEH treatment began until the end of the experiment. Procedures were identical to our previous experiment. Briefly, levers extended into the operant chamber for 10 seconds, during which rats were required to press the lever to receive reinforcement. There were 90 trials in total, with one trial occurring every 20 seconds. During this phase of training, the lights above each lever were

never illuminated. All rats were required to omit less than 10 trials in one session (approximately 5 days of training).

Then, the side bias of each rat was determined. Procedures were identical to our previous experiment. Briefly, on every trial, rats were required to respond to both levers for reward. Whichever lever the rats responded to more often during the session was determined to be ts side bias. If the rats responded equally to both levers, then the rat's initial choice on the first trial was its side bias. Following this, rats were trained on one of two rules, the visual-cue rule (light discrimination) and the spatial rule (side discrimination), depending on which task they were randomly assigned to.

Strategy set-shifting: light-side shift

Light discrimination

On the first test day, rats performing the light-side shift learned the light discrimination. This session consisted of 30-150 trials, with a trial occurring every 20 seconds. Rats were required to complete a minimum of 30 trials, such that the program would continue even if they reached criterion in less than 30 trials, after which it would end. At the beginning of each trial, one cue light above the levers was illuminated. Three seconds later, the house light was illuminated and both levers were extended into the chamber. If the rats responded to the lever above which the light is illuminated, the levers retracted, the house light stayed on for another four seconds, and one sugar pellet was delivered. If the rats responded to the opposite lever without the light illuminated, the levers retracted, the house light turned off, and no pellet was delivered. If the rat did not respond within 10 seconds of lever insertion, the levers retracted, the house light turned off, no pellet was delivered, and the response was recorded as an omission. Both cue lights were

illuminated once every two trials, with the order randomized. Criterion performance was 10 consecutive correct responses.

Shift to side discrimination

The set-shift day began with 20 reminder trials of the light discrimination, after which the correct rule shifted to the side discrimination. Here, rats were required to ignore the location of the illuminated cue lights and use a spatial-response strategy instead (ie. always respond to the left lever, regardless of which stimulus light is illuminated). The correct response was the lever opposite to each rat's side bias, which was previously determined. Criterion was 10 consecutive correct responses and animals responded to 160-180 trials, depending on whether they had 20 reminder trials or not. Rats were given a total of two days to reach criterion.

Strategy set-shifting: side-light shift

Side discrimination

Rats in the side-light group were initially trained on the side discrimination. A session consisted of 100-150 trials. All other aspects of the training procedure were identical to the training for the side discrimination in the light-side task.

Shift to light discrimination

On the day of the shift, rats had 20 reminder trials of the side discrimination before the correct rule shifted to the light discrimination. Here, animals were required to respond to the lever on top of which the stimulus light was illuminated. Here, rats were given a total of 340 trials over two days to reach criterion of 10 consecutive correct responses.

Tissue collection

All animals were euthanized the day after the final day of testing. On the day of euthanasia, animals were treated with ABI or VEH at the same time as previous days (7AM, 8AM, or 9AM) and were euthanized 4 hours later, during the same time they would have been testing in operant chambers (11AM to 2PM). Prior to euthanasia, animals were moved into the operant chamber room as normal. Animals were removed from the operant chamber room one by one in their cages, exposed to 5% isoflurane in 2L/min oxygen until they were no longer responsive to toe pinch, and euthanized via rapid decapitation using a guillotine. Two microcentrifuge tubes of trunk blood were collected and placed onto dry ice or wet ice within 3 minutes of removing the animal from the operant chamber room. One tube of trunk blood (whole blood) was stored at -70C until processing for steroid analysis. The second tube of trunk blood was centrifuged at 5080 RCF for 2 minutes for serum extraction. Serum was then stored at -70C until processing for steroid analysis. Brains were extracted and placed onto crushed dry ice within 7 minutes of removing the animal from the operant chamber room and stored at -70C.

Steroid extraction

Steroids were extracted from serum. 5µL of serum was extracted into a microtubule. 1µL of high-performance liquid chromatography (HPLC)-grade acetonitrile was added to serum (5µL) and brain samples. Next, 50µL of deuterated internal standards (testosterone-d5, corticosterone-d8, 17β-estradiol-d4, DHEA-d6, progesterone-d9) in a 50:50 mixture of HPLC-grade methanol:milliQ water (50% MeOH) were added to each sample. All samples were vortexed for 2 seconds, homogenized at 4m/s for 30 seconds using a bead mill homogenizer (Omni International Inc; Kennesaw, GA), and then centrifuged at 16,100g for 5 minutes. Afterwards,

1mL of supernatant was transferred into a 12x75mm pre-cleaned glass culture tube (washed twice with 1mL HPLC-grade MeOH), to which 500µL of HPLC-grade hexane was added. Samples were vortexed for 5 seconds and centrifuged at 3200g for 2 minutes. Hexanes were removed and samples were placed into a vacuum centrifuge (ThermoElectron SPD111V) at 60oC for 45 minutes to dry. Once samples were dried, they were resuspended in 55µL of a 1:4 mixture of HPLC-grade methanol:milliQ water (25% MeOH), vortexed for 5 seconds, and centrifuged at 3200g for 1 minute. All supernatant was then transferred to a smaller microcentrifuge tube (0.6mL) and centrifuged for a final time at 16,100g for 2 minutes. Finally, 50µL of supernatant was extracted into liquid chromatography glass insert using gel loading tips. Samples were stored in -20oC until steroid analyses.

Steroid analysis by LC-MS/MS

Steroid content was analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS), the gold standard in quantifying steroids. Samples were removed from storage in -20^{o}C and loaded into an autoinjector at 15^{o}C. 45uL of each sample was injected into a Nexera x2 UHPLC system (Shimadzu Corp., Japan). Each sample passed through an in-line filter and SecurityGuard^{TM} ULTRA C18 UHPLC guard column (2.1mm) (Phenomenex). Next, samples were separated on a Kinetex® Core-shell C18 column (2.1 x 50mm; 2.6 µm; at 40^{o}C) with two mobile phases, with mobile phase A being 0.1mM ammonium fluoride in MilliQ water and mobile phase B (MPB) being HPLC-grade methanol. The flow rate was 0.4mL/min. During loading, MPB was at 10% for 0.5 min. Then the gradient profile began at 42% MPB for 3.5 min, and then was ramped up to 60% MPB until 9.4 min. From 9.4 to 9.5 min, the gradient was 60-70% MPB, then ramped up to 98% MPB until 11.9 min. Finally, there was a column wash at

98% MPB until 13.4 min. Afterwards, MPB was returned to start conditions for 1 min. The total run time was 14.9 min per sample. Before and after each sample injection, autoinjector needle was rinsed externally with 100% isopropanol. Steroids were detected with schedule multiple reaction monitoring with two mass transitions for progesterone, corticosterone, testosterone, estradiol, and one mass transition for each internal standard. All steroid concentrations were acquired using an AB Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (AB Sciex, LLC, MA) in positive electrospray ionization mode, except for estradiol (E2), which was acquired in negative electrospray ionisation mode. See Jalabert, Ma, and Soma (2020) for retention times of each steroid.

Behavioural measures assessed

Errors to criterion, trials to criterion, omissions, and latency to respond on the initial discrimination and set-shift were calculated. Perseverative errors, regressive errors, never-reinforced errors, and number of correct reminder trials on the set-shift were calculated. See Chapter 2 for more details on each behavioural measure.

Data analysis

The primary dependent variables were errors to criterion in both initial and novel discrimination, and perseverative errors, regressive errors, and never-reinforced errors on the set-shift, because total number of errors and error type is a more sensitive measure of set-shifting performance (Tomm et al., 2022). The secondary dependent variables were trials to criterion in both initial and novel discrimination, number of correct reminder trials, and number of omissions.

Original data and log transformed data were assessed for suitability for a two-way ANOVA using the Shapiro-Wilk Test for normality and Levene's Test to measure homogenous of variance. Both original data and log transformed data had data that were not normally distributed, so we decided to use the original data. Data was analyzed using a two-way ANOVA.

A total of 5 animals were removed from analysis. One animal was removed because it was tested on the incorrect task on the initial discrimination. Four animals were removed because they performed poorly on reminder trials (scoring less than 70% accurate), which suggested that they did not learn the initial discrimination (one from light-side + VEH, three from light-side + ABI).

Results

The side-light shift is significantly more difficult than the light-side shift

Initial discrimination

Here, we determined whether androgen synthesis inhibition affected initial learning, and whether there was a difference in initial side or light discrimination learning. Neither drug nor surgery affected errors to criterion (drug: $F(1,55)=.003$, $p=.957$; task: $F(1,55)=.041$, $p=.840$; drug x task: $F(1,55)=.003$, $p=.957$) (Table 4), or trials to criterion (drug: $F(1,55)=.007$, $p=.935$; task: $F(1,55)=.854$, $p=.360$; task x drug: $F(1,55)=.007$, $p=.935$) (Table 4), suggesting that the initial discrimination, regardless of type of dimension or drug treatment, had similar levels of difficulty. There was also no effect of drug nor surgery on omissions (drug: $F(1,55)=0.044$, $p=.834$, task: $F(1,55)=2.476$, $p=.121$; task x drug: $F(1,55)=0.523$, $p=.473$) (Table 4) or latency to respond (drug: $F(1,55)=0.072$, $p=.790$; task: $F(1,55)=0.000$, $p=.993$; task x drug: $F(1,55)=1.104$, $p=.298$) (Table 4).

Set-shift to novel discrimination

Next, we determined whether inhibition of androgen synthesis or type of shift would affect performance on the set-shift to a novel discrimination. There were no effects of task or drug on the number of correct reminder trials (task: $F(1,55)=.822$, $p=.369$; drug: $F(1,55)=.397$, $p=.531$; task x drug: $F(1,55)=.033$, $p=.856$) (Table 4).

There was a significant effect of task, but not drug, on total number of errors to criterion (task: $F(1,55)=90.605$, $p>.001$; drug: $F(1,55)=.390$, $p=.535$; task x drug: $F(1,55)=.529$, $p=.470$) (Figure 4A), suggesting that regardless of drug condition, the side-light shift was more difficult than light-side shift. There was also a significant effect of task, but not drug, on perseverative errors (task: $F(1,55)=40.423$, $p<.001$; drug: ($F(1,55)=.132$, $p=.718$; task x drug: $F(1,55)=.134$, $p=.715$) (Figure 4B), regressive errors (task: $F(1,55)=34.745$, $p<.001$; drug: $F(1,55)=1.857$, $p<.179$; task x drug: $F(1,55)=1.663$, $p=.203$) (Figure 4C), and never-reinforced errors (task: $F(1,55)=30.027$, $p<.001$; drug: $F(1,55)=.258$, $p=.614$; task x drug: $F(1,55)=.258$, $p=.614$) (Figure 4D), with animals making more perseverative errors, regressive errors, and never-reinforced errors on the side-light shift compared to light-side shift. Additionally, there was an effect of task, but not drug on trials to criterion (task: $F(1,55)=124.819$, $p<.001$; drug: $F(1,55)=.310$, $p=.580$; task x drug: $F(1,55)=.273$, $p=.603$) (Table 4), with animals performing the side-light shift requiring a greater number of trials to criterion.

Next, we investigated whether these results were due to differences in motivation by assessing latency to respond and number of omissions on the set-shift. There were no significant main effect of task ($F(1,55)=2.720$, $p=.105$) or drug ($F(1,55)=0.155$, $p=.695$), or task x drug interactions ($F(1,55)=0.042$, $p=.839$) on latency to respond (Table 4). Analysis of number of

omissions found no significant main effect of task (F(1,55)=2.772, p=.102) or drug (F(1,55)=.048, p=.827), or task x drug interactions (F(1,55)=.039, p=.845) (Table 4).

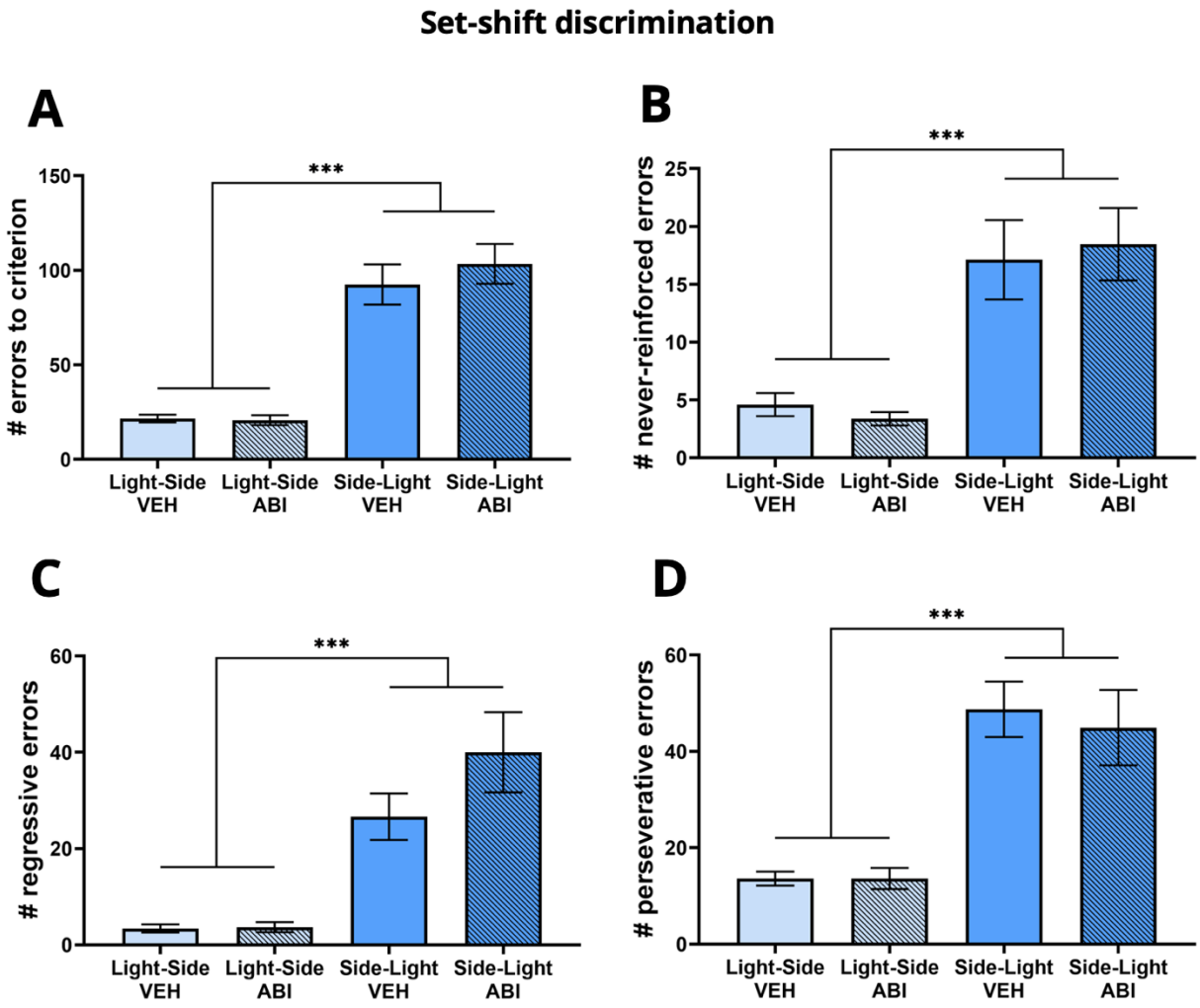

Figure 4: Effect of ABI on performance on the set-shift discrimination. There were four groups: light-side + VEH (n=14), light-side + ABI (n=11), side-light + VEH (n=16), side-light + ABI (n=15). **(A)** Errors to criterion. **(B)** Perseverative errors. **(C)** Regressive errors. **(D)** Never-reinforced errors. Bars represent mean +/- standard error of the mean (SEM). Asterisk (*) indicates significance, *** $p < .001$.

Table 4: Selected behavioural parameters for type of tasks and the effect of ABI. All data are means (SEM).

	Behavioural task			
	Light-side VEH n=14	**Light-side ABI n=11**	**Side-light VEH n=16**	**Side-light ABI n=15**
Initial discrimination				
Errors to criterion	22.80 (6.05)	21.62 (5.51)	23.75 (2.91)	23.07 (3.50)
Trials to criterion	65.13 (13.79)	76.54 (15.91)	67.31 (6.75)	76.87 (8.00)
Omissions	0.13 (0.09)	0.31 (0.21)	0.56 (0.22)	0.47 (0.19)
Latency to respond	1.29 (0.11)	1.17 (0.18)	1.13 (0.11)	1.33 (0.19)
Set-shift discrimination				
Reminder trials	16.69 (0.41)	16.38 (0.58)	17.13 (0.53)	16.73 (0.38)
Trials to criterion*	60.73 (4.50)	61.31 (6.44)	238.88 (22.42)	256.93 (21.20)
Omissions	0.40 (0.19)	0.38 (0.18)	1.69 (0.86)	1.40 (0.96)
Latency to respond	0.82 (0.11)	0.79 (0.13)	1.11 (0.14)	1.02 (0.22)

* = p<.001 when comparing task type

Steroid quantification

Serum testosterone

Here, we ensured that androgen synthesis was suppressed in animals treated with abiraterone acetate. Levels of serum testosterone were analyzed, revealing a significant effect of drug, but not task (drug: $F(1,55)=31.184$, $p<.001$; task: $F(1,55)=.073$, $p=.788$; task x drug: $F(1,55)=.054$, $p=.816$) (Figure 5A). Animals treated with abiraterone acetate had suppressed levels of serum testosterone compared to animals given vehicle.

Serum progesterone

As a secondary manipulation check to ensure ABI treatment was effective, we measured levels of progesterone. CYP17A1, the enzyme ABI inhibits, converts progestins (including progesterone) into androgens (Cools et al., 2017). Thus, if ABI treatment is effective, we should see progesterone increase in animals treated with ABI. Analysis of serum progesterone found a

significant effect of drug, but not task (drug: F(1,55)=61.734, p<.001; task: F(1,55)=0.643, p=.426; task x drug: F(1,55)=0.733, p=.396) (Figure 5B). Animals treated with abiraterone acetate had higher levels of serum progesterone.

Serum corticosterone

Here we investigated whether baseline levels of stress, as indicated by corticosterone levels, were different between our four groups. There were no effects of drug or task (drug: F(1,55)=0.000, p=.982; task: F(1,60)=1.243, p=.982; task x drug: F(1,60)=2.047, p=.158) (Figure 5C)

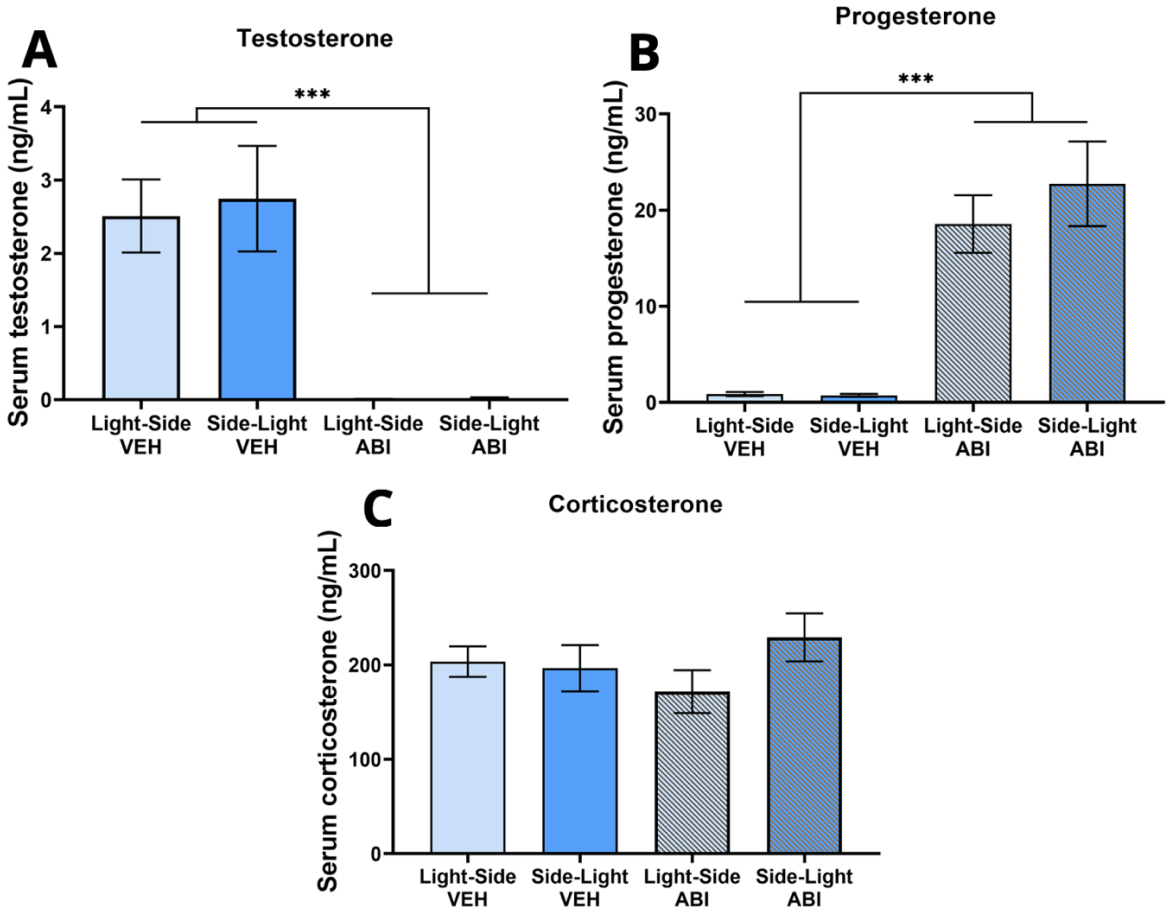

Figure 5: Effect of ABI on serum steroid levels. There were four groups: light-side + VEH (n=14), light-side + ABI (n=11), side-light + VEH (n=16), side-light + ABI (n=15). **(A)** Concentration of serum testosterone in animals. **(B)** Concentration of serum progesterone. **(C)** Concentration of serum corticosterone. All concentrations are ng/mL. Bars represent mean +/- standard error of the mean (S.E.M.). Asterisk (*) indicates significance, *** p < .001.

Serum DHEA and estradiol

DHEA and estradiol were not detected in serum of any animals.

Discussion

This current study investigated whether suppression of androgen synthesis via abiraterone acetate (ABI) treatment affects behavioural flexibility in two versions of the set-shifting paradigm, the light-side shift and side-light shift. Further, we were interested in whether there would be a difference in the effect size of ABI treatment in the light-side shift and side-light shift. Finally, we were also interested in whether we could replicate the findings from my previous study showing the side-light shift is more difficult than the light-side shift. We found no effect of ABI treatment on any behavioural parameters measured, on both side-light and light-side set-shifting paradigm. We did, however, replicate previous findings that the side-light shift is more difficult than the light-side shift.

ABI did not improve performance on both versions of the set-shifting paradigm

Testosterone plays a role in behavioural flexibility. In 1973, Rogers demonstrated that systemic injections of testosterone in male chickens impaired behavioural flexibility by increasing perseveration. Similarly, systemic treatment of testosterone in male adolescent rodents impaired performance on multiple versions of the strategy set-shifting paradigm (Wallin & Wood, 2015). Furthermore, both castrated mice and chicks rescued with testosterone were more likely to perseverate on the relevant stimulus and have difficulty shifting to an irrelevant stimulus, suggesting testosterone treatment impairs behavioural flexibility (Archer, 1974).

Inhibiting the actions of androgens via antiandrogen or ABI treatment improves behavioural flexibility. Castrated male chickens and male chickens injected with antiandrogen cyproterone acetate were less perseverative than control animals, and similar to female chickens, in a food searching task that measured an animal's ability to flexibly switch between a preferred and non-preferred seed (Rogers, 1973). Additionally, rats given cyproterone acetate improved flexibility on both the light-side and side-light shift, as indicated by a lower number of trials to criterion (Thompson & Wright, 1979). Since cyproterone acetate exerts its effects by blocking androgen receptors, these studies show that reducing androgen signaling improves behavioural flexibility.

This effect was further highlighted by Tomm et al., (2022), who found that ABI improved behavioural flexibility on both reversal learning and strategy set-shifting (light-side shift), as indicated by a significantly decreased number of perseverative errors and total number of errors to criterion, respectively. This effect was present regardless of whether animals were intact or gonadectomized. The authors suggested that this may be driven by changes in dopamine signaling in the mesocorticolimbic system, which is important for behavioural flexibility. Expression of tyrosine hydroxylase (Th), the rate-limiting enzyme of dopamine production (Daubner, Le, & Wang, 2011), was increased in the medial prefrontal cortex and tended to decrease in the nucleus accumbens core. The authors suggested that this change in Th expression, and thus prefrontal dopaminergic activity, may be a mechanism through which ABI altered set-shifting behaviour.

Despite previous research showing a modulating effect of the absence of testosterone on behavioural flexibility, we found no effect of ABI treatment on both the light-side and side-light set-shifting paradigm. This was not due to manipulation failure. Using liquid chromatography tandem mass spectrometry (LCMS/MS), we demonstrated that ABI treatment was successful.

Animals treated with ABI had negligible levels of serum testosterone, whereas animals treated with vehicle had much higher levels of detected testosterone.

While serum testosterone levels were reduced, we did not measure ABI in the brain to ensure that Abi crossed the blood brain barrier successfully. However, there are several lines of evidence showing it is likely that ABI was in the brain. First, ABI can cross the blood brain barrier, as demonstrated by a study that found that ABI peaks in the brain, along with various other tissues, 4 hours after oral treatment (European Medicines Agency, 2011). Next, Tomm et al., (2022) demonstrated that 4 days of oral treatment with ABI at a dose of 40mg/kg reduced testosterone levels in both serum and brain tissue. Furthermore, ABI was detected in both serum and brain tissue. Since our animals received 40mg/kg of ABI daily for 5 days prior to behavioural testing, and daily during behavioural testing, it is reasonable to believe that ABI did reach the brain in our study.

Nevertheless, despite our manipulation being successful, we were not able to replicate the findings by Tomm et al., (2022). Taking a closer look at the data may help reconcile these differences. Tomm et al., found no differences in initial light discrimination learning, with sham animals requiring ~45 trials and ~10 errors to meet criterion, while animals in the present study required ~70 trials and ~20 errors to meet criterion. On average, it appears animals in the present study had more difficulty learning the light discrimination. Further, with regards to the shift to side discrimination, animals given ABI in both studies performed similarly, whereas our control animals required fewer trials to criterion and made fewer errors to criterion, indicating that they may have, on average, performed better than Tomm et al.,'s sham-vehicle animals.

This difference in ability to learn the initial discrimination may be attributed to several explanations. First, animals in the two studies may have had some baseline differences that led to

an impaired ability to learn the light discrimination, such as visual ability and ability to ignore irrelevant cues. Ultimately, an impairment in learning one strategy could subsequently affect the learning of a novel strategy. Second, it could also point to an external factor specific to one of the studies that may have affected learning.

One external factor is the house light position. In Tomm et al.,'s study, the house light was positioned directly opposite to the wall where the levers and cue-lights were located. In the current study, the house light was located at the front left corner, behind the wall where cue-lights are located. During every trial, one cue-light would turn on to signal the correct lever, and 3 seconds later, both levers would extend, and the house light would illuminate. This is an issue because rats may become distracted by the house light, leading to confusion about which light source is the correct signal. This could have impaired performance on the light discrimination, which could have affected the subsequent shift. This confusion can explain why it may have been more difficult for animals in the present study to learn the initial light discrimination compared to animals in Tomm et al.,'s study.

If animals in our present study had more difficulty learning the light discrimination, it is possible it could affect performance on the subsequent shift, leading to the differences seen between our study and Tomm et al.,'s study. One explanation for our control animals performing similarly to our ABI animals includes concepts from a weighted attention model (Aluisi, Rubinchik, & Morris, 2018).

Aluisi, Rubinchik, and Morris (2018) suggested a weighted attention model could quantify and explain choices on a digging intra-dimensional/extra-dimensional set-shifting paradigm. They explain that during each trial, rats will assign a certain weight for each dimension (visual-cue light or spatial position, i.e., side) based on their experience of reward or no reward, and

ultimately make their subsequent decisions by comparing the average weights assigned to each dimension. During the set-shift, the weight associated with the initial relevant dimension was high compared to the novel dimension, but as performance improve on the novel discrimination, this difference decreases.

Pulling concepts from this model, in our set-shifting paradigm, it seems likely that as rats learn the discriminations through trial-and-error, they will assign weights to each dimension to help them succeed in later trials. Presumably, if a strategy is more difficult for a rat to learn, it may be because the rat is not assigning a high enough weight to that dimension. Thus, when the set-shift occurs, if the animal did not assign a high weight to the initially relevant dimension, it will presumably be easier to notice an incorrect choice and assign a higher weight to the novel dimension faster.

If a more difficult initial discrimination makes the subsequent shift easier to learn, it is possible the shift was too easy and there was a floor effect, which would make any effect by ABI difficult to detect. Indeed, one of the reasons we wanted to make the set-shifting task more difficult was to increase the small effect size of ABI found by Tomm et al., (~6 errors to criterion between vehicle and ABI animals). A small effect size and floor effect caused by a more difficult initial discrimination can certainly explain the discrepancies between our study.

Furthermore, a small effect would ultimately require a larger sample size to detect; perhaps the sample sizes of 16 we used in our study was not enough to detect an effect. In fact, Tomm et al., used 19-20 rats per group, whereas we used 16. This discrepancy between sample sizes could also contribute to our conflicting results.

Alternatively, another reason for the discrepancy between our studies may be due to long-term effects of isoflurane exposure and/or surgical stress. Tomm et al.,'s animals received either

gonadectomy or sham-gonadectomy, and thus inhalational isoflurane, whereas my animals were not exposed to isoflurane or surgical stress before behavioural testing. Isoflurane is a known stressor and there is evidence it has chronic effects on animals. Geddes et al., (2021) demonstrated that rats given a sham craniectomy under inhalational isoflurane had suppressed levels of circulating systemic testosterone 1-29 days post-surgery, with 29 days being the final day of measurement. While it is difficult to distinguish whether this suppression is due to stress from the sham surgery or from isoflurane exposure, or both, there is evidence suggesting it may partially be due to isoflurane exposure. Xu et al. (2012) found that inhalational isoflurane exposure can suppress systemic levels of follicle-stimulating hormone (FSH), a hormone that regulates testosterone levels, and testosterone.

Given this notion that isoflurane exposure can alter levels of testosterone, potentially by altering levels of FSH, it is reasonable to believe there might be downstream effects of altering systemic levels of testosterone and FSH, or direct effects of isoflurane that may influence testosterone levels or function in the brain. Indeed, our current animals had higher levels of serum testosterone compared to Tomm et al.,'s intact animals, suggesting there may have been some long-term effects of surgical stress or isoflurane exposure. This, in addition to any other chronic effects, could explain the discrepancies between our two studies.

Finally, we cannot exclude the possibility that our results are accurate, and the effect found by Tomm et al., is not a real effect, and represents a type 1 error. In any study using a 95% confidence level like Tomm et al.,'s study, there is a 5% chance of getting a type 1 error, in which an effect is found where there is no real effect. If this was the case, then our findings would indicate that inhibition of androgen synthesis does not improve behavioural flexibility. However, this seems unlikely, given the pattern of results from previous studies that have

demonstrated that suppression of the actions of testosterone via antiandrogens improves set-shifting (Thompson & Wright, 1979; Rogers, 1973). Additionally, Tomm et al., also demonstrated an effect of ABI on reversal learning, another form of behavioural flexibility, which further illustrates a relationship between the absence of androgen signaling and behavioural flexibility. Further research is necessary to clarify this and determine whether inhibiting androgen synthesis improves behavioural flexibility.

We also did not find any effect of ABI treatment on performance on the side-light shift. This contrasts with Thompson & Wright (1979), who did find an effect of cyproterone acetate, an antiandrogen, on the side-light shift. One reason for this might be due to the location of the house light and how it may have impacted the difficulty of the light discrimination. There were many rats in the present study in both ABI and vehicle groups who did not learn the light discrimination on the shift even after receiving the maximum number of trials allotted for the task. Therefore, there may have been a potential ceiling effect, making any potential effect difficult to detect. Alternatively, it is possible that the side-light shift is not sensitive to the effects of testosterone. Thus, more research is required to determine whether eliminating testosterone affects performance on the side-light shift.

The side-light shift is significantly more difficult than the light-side shift

Our findings indicate that the side-light shift is more difficult for rats to perform in operant chambers than the light-side shift, a finding that is consistent with the study in chapter 1. Although this result contrasts with Floresco et al., (2008), who found that the light-side shift was more difficult, it remains consistent with previous findings from Wallin & Wood (2015) and Bercovici et al., (published), who both demonstrated that the side-light shift is more difficult.

As we have previously discussed, one reason the side-light shift may be more difficult is because it may involve a greater amount of cognitive load to complete. The confusing position of the house light in the current study may act as an additional irrelevant cue animals must learn to ignore. Thus, when animals shift from the side-light discrimination, in addition to learning to respond to the cue-lights, they must also learn to ignore the irrelevant house light. Since animals shifting from side-light do not have to learn to ignore the house light, it can be argued that animals shifting from side-light discrimination will have a greater cognitive load.

While we were able to replicate the finding that the side-light shift is more difficult, we were not able to replicate the finding that for naïve rats, the initial light discrimination is more difficult to learn than the initial side discrimination. This adds to the list of inconsistent data on this front. Several studies, including our study in chapter 1, have found that in both the maze and automated version of the set-shifting paradigm, the visual-cue dimension (including light discrimination) is more difficult to learn than the spatial dimension (including side discrimination) for adult male rats (Floresco, Block, & Tse, 2008; Floresco et al., 2006; Ragozzino, 2002). On the other hand, Wallin & Wood (2015) demonstrated the opposite effect – the light discrimination was more difficult to learn than side discrimination for naïve adolescent male rats. However, we previously explained that adolescent rats might have innate differences from adult rats, which may ultimately render a direct comparison inappropriate.

There are a few reasons why we may have found conflicting findings in terms of initial discrimination learning. In our first study, we found cohort effects on initial discrimination learning that showed animals in the second cohort of 16 performed significantly better on the light discrimination than animals in the first cohort of 16. Thus, we cannot be sure if our finding

that the initial light discrimination is easier to learn is being driven by cohort effects of an actual difference. Further research will be required to tease this apart.

Additionally, the cohort effects from our previous study and the inconsistent data yielded by multiple studies and multiple labs suggests that rats may exhibit large individual variability in their ability to learn and respond to the light discrimination. Indeed, taking a closer look at the data in both of our studies thus far will show that on the initial light discrimination, trials to criterion ranges from 10-147 in our previous study, and 10-150 in this current study, with several animals not meeting criterion even after the maximum 150 trials on the initial discrimination. This wide range clearly demonstrates large individual differences in learning the initial light discrimination. It is unclear whether these differences are in vision, innate bias towards a certain type of strategy, ability to ignore irrelevant information, or ability to remember relevant information. While individual differences are expected in any study involving multiple subjects, if these differences are large and we have sample sizes of 8-16 (which is typical of animal behaviour studies), having a small number of outliers could easily skew the data.

In conclusion, we found that suppression of testosterone levels does not affect behavioural flexibility on both the light-side and side-light set-shifting paradigm. Additionally, we found that the side-light shift is more difficult than the light-side shift for animals treated with ABI and vehicle.

Chapter 4: General Discussion, Limitations, Future Directions, and Conclusions

General discussion

This current thesis consists of two experiments. The first experiment explored different ways to make the strategy set-shifting paradigm more difficult for rodents. We found that the side-light shift was more difficult than the light-side shift, which may be due to the greater amount of cognitive load and effort the shift requires. However, there was no effect of minimum training on the initial discrimination or reminder trials on the subsequent shift. This might be because the amount of overtraining our animals received through an increase in minimum training or reminder trials was not enough to produce any meaningful effect, unlike previous studies. The second experiment investigated whether abiraterone acetate (ABI), a drug that inhibits testosterone synthesis by targeting CYP17A1, improves performance on the light-side and side-light set-shifting paradigm. While we were able to replicate our previous finding that the side-light shift was more difficult, we found no effect of ABI on performance on both versions of the set-shifting paradigm. This was not the result of manipulation failure, as our data show that ABI was effective at eliminating serum testosterone. However, it may be due to differences between our experiments, such as the house light position and exposure to isoflurane, the difficulty of detecting a small but real effect, or the possibility that Tomm et al.,'s findings represent a type 1 error.

Limitations

There were a couple limitations in our experiments that should be addressed for future studies. First, the position of the house light was problematic for reasons discussed previously. Second, the lights in the colony room in which rats were housed were brighter than in previous

experiments in our lab. The unfiltered lights were approximately 190 lux whereas the same lights with the filter were approximately 85 lux (Lo, unpublished). Long Evans rats who were exposed to a light intensity of 500 lux continuously demonstrated differences in retinal concentration of certain amino acids, suggesting photoreceptors are unable to synthesize it (Wasowicz et al., 2002). Whether these differences translate to actual vision impairments is unknown. While a brightness of 500 lux is much brighter than the 190 lux in our current experiments, Sprague-Dawley rats exposed to cyclic light intensities of 200 lux present with retinal damage (Liu et al., 2019). It is possible that an intensity of 190 lux could have altered the retina of our rats. Furthermore, Blom et al., (1995) demonstrate that pigmented rats (a group which includes Long Evans rats) prefer environments with a low light intensity (below 100 lux) compared to those with higher intensity (100 lux to 380 lux). Therefore, future studies should ensure the lighting in the room where rats are housed are below 100 lux to prevent any possible vision impairments or stress caused by brighter lights.

A few other factors to consider for future experiments are breeding facility and housing facility. Due to reasons outside of our control, we were unable to conduct our experiments in the same housing facility or use animals from the same breeding facility as the study by Tomm et al., (2022). These changes can be problematic because each housing and breeding facility can have differences in husbandry practices such as water decontamination methods, diet composition, autoclaving and irradiation methods, and the addition of new dams into a colony, that could affect gut microbiota composition (Franklin & Ericsson, 2018). Differences in gut microbiota have been shown to influence short- and long-term memory and cognitive flexibility in mice (Magnusson et al., 2015), and depression-like behaviour in rats (Liu et al., 2020). Thus, it would

be important to ensure that animals from future studies originate from the same breeding facility and are housed in the same housing facility, where possible.

Future directions

Findings from our studies have conflicted with previous research showing that inhibiting androgen production or action improves behavioural flexibility. Future studies should clarify this effect, specifically by looking at performance on the strategy set-shifting paradigm. In this study, researchers should be careful with operant chamber design, ensuring that the house light is in an appropriate location that will not confuse an animal during the light discrimination. If the absence of androgens is shown to improve behavioural flexibility, a next step could investigate the role of locally produced testosterone on behavioural flexibility by performing intracranial injections of antiandrogens or ABI directly into the mesocorticolimbic system of gonadectomized animals. Additionally, it would be interesting to rescue animals given ABI with testosterone treatment to see how set-shifting performance would be affected. These findings could have future implications on treating psychiatric and neurological illnesses characterized by executive function disorders, such as depression, schizophrenia, and autism spectrum disorder (Abel, Drake & Goldstein, 2010; Werling & Geschwind, 2013; Eid, Gobinath, & Galea, 2019). Alternatively, one could explore the possibility that these effects, if found, could be due to testosterone, estrogens, or both steroids binding to androgen receptors. This is because in the male rat brain, testosterone can be locally converted to estrogens by aromatase, and there is also evidence suggesting 17 β-estradiol (E2) can bind to androgen receptors (Yeh et al., 1998).

Furthermore, given that estrogens are produced locally in the female rat brain and the synthesis of estrogens requires the conversion of androgens to estrogens by aromatase suggests

that testosterone may be, at some level, present in the female rat brain (Hojo & Kawato, 2018). Since 17 β-estradiol treatment alters extra-dimensional set-shifting performance in female rats (Lipatova et al., 2016), it would be fascinating to investigate whether this effect is due to androgens or estrogens, or both. It would also be important to determine the role of locally produced estrogens on behavioural flexibility in female rats, as women are disproportionately affected by mental health illnesses that present with sex differences and executive dysfunction such as depression and anxiety (Salk, Hyde, & Abramson, 2017; Altemus, Sarvaiya, & Epperson, 2016).

It would also be interesting to expand from behavioural flexibility and investigate the role of androgens or estrogens in other executive functions that exhibit sex differences, including risky decision making and impulsivity. For example, risky decision making can be assessed by the probabilistic discounting task, which requires animals to choose between a small but certain reward or a large but uncertain reward, where the probability for reward delivery decreases over time (Gilbert et al., 2011). This task has been shown to have sex differences and be sensitive to the effects of testosterone (Islas-Preciado et al., 2020), suggesting this may be a future avenue of interest.

Additionally, a delayed discounting task, which requires animals to choose between a small but immediate reward and a large but delayed reward, has demonstrated that impulsivity may be mediated by the presence of androgens (Hernandez et al., 2020). Here, female rats and gonadectomized male rats made a greater number of impulsive choices compared to intact male rats. Thus, it would be interesting to determine the role of neurally produced androgens and estrogens in impulsive behaviour.

Conclusions

This thesis aimed to create a more difficult strategy set-shifting paradigm and to investigate the effects of androgen synthesis inhibition on two versions of the strategy set-shifting paradigm. In both experiments, the side-light shift was more difficult than the light-side shift, indicating that this manipulation successfully increased the difficulty of the task. There was no effect of minimum amount of training on the initial light discrimination or the presence or absence of reminder trials on performance on the strategy set-shifting paradigm. Additionally, there was no effect of androgen synthesis inhibition on either the light-side shift or the side-light shift. The current data provide insight into the strategy set-shifting task and the potential role of androgens on behavioural flexibility, suggesting that additional research is necessary.

References

Abel, K. M., Drake, R., & Goldstein, J. M. (2010). Sex differences in schizophrenia, *International Review of Psychiatry,* 22(5), 417-428.

Altemus, M., Sarvaiya, N., & Neill Epperson, C. (2014). Sex differences in anxiety and depression clinical perspectives. *Frontiers in Neuroendocrinology*, 35(3), 320–330.

Aluisi, F., Rubinchik, A., & Morris, G. (2018). Animal Learning in a Multidimensional Discrimination Task as Explained by Dimension-Specific Allocation of Attention. *Frontiers in Neuroscience*, 12, 356.

Archer, J. (1974). The effects of testosterone on the distractibility of chicks by irrelevant and relevant novel stimuli, *Animal Behaviour,* 22, 397-404.

Aubele, T., & Kritzer, M. F. (2011). Gonadectomy and hormone replacement affects in vivo basal extracellular dopamine levels in the prefrontal cortex but not motor cortex of adult male rats. *Cerebral cortex*, 21(1), 222–232.

Aubele, T., & Kritzer, M. F. (2012). Androgen influence on prefrontal dopamine systems in adult male rats: localization of cognate intracellular receptors in medial prefrontal projections to the ventral tegmental area and effects of gonadectomy and hormone replacement on glutamate-stimulated extracellular dopamine level. *Cerebral cortex*, 22(8), 1799–1812.

Banks, W. A. (2012). Brain meets body: the blood-brain barrier as an endocrine interface, *Endocrinology,* 153(9), 4111-4119.

Baulieu, E. E., & Robel, P. (1990). Neurosteroids: a new brain function?. *The Journal of steroid biochemistry and molecular biology,* 37(3), 395–403.

Birrell, J. M., & Brown, V. J. (2000). Medial frontal cortex mediates perceptual attentional set shifting in the rat, *The Journal of Neuroscience,* 20(11), 4320-4324.

Blom, H. J. M., Van Tintelen, G., Baumans, V., Van Decn Broek, J., & Beynen, A. C. (1995). Development and application of a preference test system to evaluate housing conditions for laboratory rats, *Applied Animal Behaviour Science,* 43(4), 279-290.

Bromberg-Martin, E. S., Matsumoto, M., & Hikosaka, O. (2010). Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron*, 68(5), 815–834.

Cai, H., Cao, T., Zhou, X., Yao, J. K. (2018). Neurosteroids in schizophrenia: pathogenic and therapeutic implications, *Frontiers in Psychiatry*, 9(73), DOI: 10.3389/fpsyt.2018.00073

Chen, J. (2017). Empathy for distress in humans and rodents, *Neuroscience Bulletin,* 34(1), 216-236.

Cools, M., Claahsen-van der Grinten, H. L., De Baere, E., Callens, & N., Dessens, A. B. (2017) 4.03 – Genetic defects of female sexual differentiation, *Hormones, Brain and Behaviour (Third Edition),* 4, 105-134.

Cooper, S., Robison, A. J., Mazei-Robison, M. S. (2017). Reward circuitry in addiction, *Neurotherapeutics,* 14, 687-697.

Daubner, S. C., Le, T., & Wang, S. (2012). Tyrosine hydroxylase and regulation of dopamine synthesis, *Archives of Biochemistry and Biophysics,* 508(1), 1-12.

Del Arco, A., Park, J., Wood, J., Kim, Y., & Moghaddam, B. (2017). Adaptive encoding of outcome prediction by prefrontal cortex ensembles supports behavioural flexibility, *The Journal of Neuroscience,* 37(35), 8363-8373.

Dias, R., & Aggleton, J. P. (2008). Effects of selective excitotoxic prefrontal lesions on acquisition of nonmatching- and matching-to-place in the T-maze in the rat: differential

involvement of the prelimbic-infralimbic and anterior cingulate cortices in providing behavioural flexibility, *European Journal of Neuroscience*, 12(12), 4457-4466.

Durstewitz, D., Vittoz, N. M., Floresco, S. B., Seamans, J. K. (2010). Abrupt transitions between prefrontal neural ensemble states accompany behavioural transitions during rule learning, *Neuron,* 66(3), 438-338.

Eid, R. S., Gobinath, A. R., & Galea, L. A. M. (2019). Sex differences in depression: insights from clinical and preclinical studies, *Progress in Neurobiology,* 176, 86-102.

Emanuelsson, I., Almokhtar, M., Wikvall, K., Grönbladh, A., Nylander, E., Svensson, A. L., Svenningsen, Å. F., & Norlin, M. (2018). Expressedion and regulation of CYP17A1 and 3β-hydroxysteroid dehydrogenase in cells of the nervous system: potential effects of vitamin D on brain steroidogenesis, *Neurochemistry International,* 113, 46-55.

European Medicines Agency. (2011). Assessment Report for Zytiga (Abiraterone). *Committee for Medicinal Products for Human Use (CHMP)*

Farina, F. R., Burke, T., Coyle, D., Jeter, K., McGee, M., O'Connell, J., Taheny, D., & Commins, S. (2015). Learning efficiency: the influence of cue salience during spatial navigation, *Behavioural Processes,* 116, 17-27.

Fester, L., & Rune, G. M. (2021). Sex neurosteroids: hormones made by the brain for the brain, *Neuroscience Letters,* 753:135849.

Floresco, S. B., Block, A. E., & Tse, M. T. L. (2008). Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure, *Behavioural Brain Research,* 190, 85-96.

Floresco, S. B., Ghods-Sharifi, S., Vexelman, C., & Magyar, O. (2006a). Dissociable roles for the nucleus accumbens core and shell in regulating set shifting, *The Journal of Neuroscience,* 26(9), 2449-2457.

Floresco, S. B., Magyar, O., Ghods-Sharifi, S., Vexelman, C., & Tse, M. T. L. (2006b). Multiple dopamine receptor subtypes in the medial prefrontal cortex of the rat regulate set-shifting, *Neuropsychopharmacology,* 31, 297-309.

Franklin, C. L., & Ericsson, A. C. (2017). Microbiota and reproducibility of rodent models. *Lab animal,* 46(4), 114–122.

Garner, J. P., Thogerson, C. M., Würbel, H., Murray, J. D., & Mench, J. A. (2006). Animal neuropsychology: validation of the intra-dimensional extra-dimensional set shifting task for mice, *Behavioural Brain Research,* 173, 53-61.

Geddes, R. I., Kapoor, A., Hayashi, K., Rauh, R., Wehber, M., Bongers, Q., Jansen, A. D., Anderson, I. M., Farquhar, G., Vadakkadath-Meethal, S., Ziegler, T. E., & Atwood, C. S. (2021). Hypogonadism induced by surgical stress and brain trauma is reversed by human chorionic gonadotropin in male rats: A potential therapy for surgical and TBI-induced hypogonadism?. *Endocrinology, diabetes & metabolism*, 4(3), e00239.

Giatti, S., Diviccaro, S., Garcia-Segura, L. M., & Melcangi, R. C. (2019). Sex differences in the brain expression of steroidogenic molecules under basal conditions and after gonadectomy. *Journal of neuroendocrinology*, 31(6), e12736.

Gilbert, R. J., Mitchell, M R., Simon, N. W., Bańuelos, C., Setlow, B., & Bizon, J. L. (2011). Risk, reward, and decision-making in a rodent model of cognitive aging, *Frontiers in Neuroscience*, 5, 144.

Goascogne, C. L., Robel, P., Gouézou, M., Sananès, N., Baulier, E. E., & Waterman, M. (1987). Neurosteroids: cytochrome P-450scc in rat brain, *Science*, 237(4819), 1212-1215.

Haluk, D. M., & Floresco, S. B. (2009). Ventral striatal dopamine modulation of different forms of behavioural flexibility, *Neuropsychopharmacology,* 34, 2041-2052.

Hernandez, C. M., Orsini, C., Wheeler, A. R., Ten Eyck, T. W., Betzhold, S. M., Labiste, C. C., Wright, N. G., Setlow, B., & Bizon, J. L. (2020). Testicular hormones mediate robust sex differences in impulsive choice in rats, *eLife,* 9, e58604.

Hojo, Y., & Kawato, S. (2018). Neurosteroids in Adult Hippocampus of Male and Female Rodents: Biosynthesis and Actions of Sex Steroids. *Frontiers in Endocrinology*, 9, 183.

Jalabert, C., Ma, C., & Soma, K. K. (2021). Profiling of systemic and brain steroids in male songbirds: Seasonal changes in neurosteroids. *Journal of neuroendocrinology*, 33(1), e12922.

Kazuyoshi, T., Kazuyoshi, U., Minoru, T., Kohchi, C., Lea, R. L. (1999). Neurosteroid biosynthesis in vertebrate brains, *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology, and Endocrinology,* 124(2), 121-129.

Kesner, R. P., & Churchwell, J. C. (2011). An analysis of rat prefrontal cortex in mediating executive function. *Neurobiology of learning and memory*, 96(3), 417–431.

King S. R. (2008). Emerging roles for neurosteroids in sexual behavior and function. *Journal of andrology*, 29(5), 524–533.

Kirschmann, E. K., Pollock, M. W., Nagarajan, V., & Torregrossa, M. M. (2019). Development of working memory in the male adolescent rat. *Developmental cognitive neuroscience*, 37, 100601.

Kritzer M. (2004). The distribution of immunoreactivity for intracellular androgen receptors in the cerebral cortex of hormonally intact adult male and female rats: localization in pyramidal neurons making corticocortical connections. Cerebral cortex (New York, N.Y. : 1991), 14(3), 268–280.

Li, R., Ma, X., Wang, G., Yang, J., & Wang, C. (2016). Why sex differences in schizophrenia?. Journal of translational neuroscience, 1(1), 37–42.Liu, S., Guo, R., Liu, F., Yuan, Q., Yu, Y., & Ren, F. (2020). Gut Microbiota Regulates Depression-Like Behavior in Rats Through the Neuroendocrine-Immune-Mitochondrial Pathway. *Neuropsychiatric Disease and Treatment,* 16, 859–869.

Lipatova, O., Wiener, N., Andrews, K., Kirshenbaum, A. P., Green, J. T., & Toufexis, D. J. (2016). 17β-estradiol replacement in ovariectomized female rats slows set 1 dorsolateral striatial-dependent learning and enhances learning of set 2 in an extradimensional set-shifting paradigm. *Behavioral Neuroscience*, 130(1), 44–49.

Liu, X., Zhou, Q., Lin, H., Wu, J., Wu, Z., Qu, S., & Bi, Y. (2019). The Protective Effects of Blue Light-Blocking Films With Different Shielding Rates: A Rat Model Study. *Translational Vision Science & Technology*, 8(3), 19.

Low, K. L., Ma, C., & Soma, K. K. (2017). Tyramide Signal Amplification Permits Immunohistochemical Analyses of Androgen Receptors in the Rat Prefrontal Cortex. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 65(5), 295–308.

Low, K. L., Tomm, R. J., Ma, C., Tobiansky, D. J., Floresco, S. B., & Soma, K. K. (2020). Effects of aging on testosterone and androgen receptors in the mesocorticolimbic system of male rats. *Hormones and behavior*, 120, 104689.

Magnusson, K. R., Hauck, L., Jeffrey, B. M., Elias, V., Humphrey, A., Nath, R., Perrone, A., & Bermudez, L. E. (2015). Relationships between diet-related changes in the gut microbiome and cognitive flexibility. *Neuroscience*, 300, 128–140.

McHenry, J., Carrier, N., Hull, E., & Kabbaj, M. (2014). Sex differences in anxiety and depression: role of testosterone, *Frontiers in Neuroendocrinology,* 35(1), 42-57.

Mensah-Nyagan, A. G., Do-Rego, J. L., Beaujean, D., Luu-The, V., Pelletier, G., & Vaudry, H. (1999). Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system, *Pharmacological Reviews*, 51(1)- 63-82.

Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews*, 32(1), 81–151.

Norman, D. A., & Bobrow, D. G. (1976). On the analysis of performance operating characteristics. Psychological Review, 83(6), 508–510. DOI: 10.1037/0033-295X.83.6.508

Orth, M., & Bellosta, S. (2012). Cholesterol: its regulation and role in central nervous system disorders, *Cholesterol,* 292598.

Owen, A. M., Roberts, A. C., Hodges, J. R., Summers, B. A., Polkey, C. E., & Robbins, T. W. (1993). Contrasting mechanisms of impaired attention set-shifting in patients with frontal lobe damage or Parkinson's disease, *Brain,* 116(5), 1159-1175.

Paul, S. M., & Purdy, R. H. (1992). Neuroactive steroids. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 6(6), 2311–2322.

Pelletier, G., Luu-The, V., & Labrie, F. (1995). Immunocytochemical localization of type 1 17β-hydroxysteroid dehydrogenase in the rat brain, *Brain Research,* 704(2), 233-239.

Peters-Scheffer, N., Didden, R., Sigafoos, J., Green. V. A., & Korzilius, H. (2013). Behavioural flexibility in children with autism spectrum disorder and intellectual disability, *Research in Autism Spectrum Disorders*, 7(6), 699-709.

Ragozzino, M. E. (2002). The effects of dopamine D1 receptor blockade in the prelimbic-infralimbic areas on behavioral flexibility, *Learning and Memory,* 9, 18-28.

Ragozzino, M. E., Detrick, S., & Kesner, R. P. (1999). Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioural flexibility for place and response learning, *The Journal of Neuroscience*, 19(11), 4585-4594.

Ragozzino, M. E., Kim, J., Hassert, D., Minniti, N., & Kiang, C. (2003). The contribution of the rat prelimbic-infralimbic areas to different forms of task switching, *Behavioral Neuroscience*, 117(5), 1054-1065.

Ratner, M. H., Kumaresan, V., & Farb, D. H. (2019). Neurosteroid Actions in Memory and Neurologic/Neuropsychiatric Disorders. *Frontiers in endocrinology*, 10, 169.

Rich, E. L., & Shapiro, M. L. (2007). Prelimbic/infralimbic inactivation impairs memory for multiple task switches, but not flexible section of familiar tasks, *Journal of Neuroscience*, 27(17), 4747-4755.

Rich, E. L., & Shapiro, M. L. (2009). Rat prefrontal cortical neurons selectively code strategy switches, *Journal of Neuroscience,* 29(22), 7208-7219.

Rogers, L. J. (1974). Persistence and search influenced by natural levels of androgens in young and adult chickens, *Physiology & Behaviour,* 12(2), 197-204.

Ruiz-Cortés, Z. T. (2012). Gonadal sex steroids: production, action, and interactions in mammals. In (Ed.), Steroids – From Physiology to Clinical Medicine. IntechOpen.

Salk, R. H., Hyde, J. S., & Abramson, L. Y. (2017). Gender differences in depression in representative national samples: Meta-analyses of diagnoses and symptoms. *Psychological Bulletin*, 143(8), 783–822.

Sol, D., Lapiedra, O., & González-Lagos, C. (2013). Behavioural adjustments for a life in the city, *Animal Behaviour,* 85(5), 1101-1112.

Soma, K. K., Scotti, M. A., Newman, A. E., Charlier, T. D., & Demas, G. E. (2008). Novel mechanisms for neuroendocrine regulation of aggression. *Frontiers in neuroendocrinology*, 29(4), 476–489.

Strömstedt, M., & Waterman, M. R. (1995). Messenger RNAs encoding steroidogenic enzymes are expressed in rodent brain, *Molecular Brain Research,* 34(1), 75-88.

Terra, H., Bruinsma, B., F de Kloet, S., van der Roest, M., Pattij, T., & Mansvelder, H. D. (2020). Prefrontal cortical projection neurons targeting dorsomedial striatum control behavioral inhibition, *Current Biology,* 30(21), 4188-4200.

Thompson, W. R., & Wright, J. S. (1979). "Persistence" in rats: Effects of testosterone, *Psychobiology,* 7, 291–294.

Tobiansky, D. J., Korol, A. M., Ma, C., Hamden, J. E., Jalabert, C., Tomm, R. J., & Soma, K. K. (2018a). Testosterone and Corticosterone in the Mesocorticolimbic System of Male Rats: Effects of Gonadectomy and Caloric Restriction. *Endocrinology*, 159(1), 450–464.

Tobiansky, D. J., Wallin-Miller, K. G., Floresco, S. B., Wood, R. I., & Soma, K. K. (2018b). Androgen Regulation of the Mesocorticolimbic System and Executive Function. Frontiers in endocrinology, 9, 279.

Tomm, R. J., Seib, D. R., Kachkovski, G. V., Schweitzer, H. R., Tobiansky, D. J., Floresco, S. B., & Soma, K. K. (2022). Androgen synthesis inhibition but not gonadectomy reduces

persistence during strategy set-shifting and reversal learning in male rats, *Journal of Neuroendocrinology,* Accepted.

Triemstra, J. L., Sato, S. M., & Wood, R. I. (2008). Testosterone and nucleus accumbens dopamine in the male Syrian hamster. *Psychoneuroendocrinology*, 33(3), 386–394.

Uddin, L.Q. (2021). Cognitive and behavioural flexibility: neural mechanisms and clinical considerations. *Nature Reviews Neuroscience*, 22, 167–179.

Wallin, K. G., & Wood, R. I. (2015). Anabolic-androgenic steroids impair set-shifting and reversal learning in male rats. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology*, 25(4), 583–590.

Waltz J. A. (2017). The neural underpinnings of cognitive flexibility and their disruption in psychotic illness. *Neuroscience*, 345, 203–217. DOI: 10.1016/j.neuroscience.2016.06.005

Wang, M. (2011). Neurosteroids and GABA-A receptor function, *Frontiers in Endocrinology,* 2, 44.

Wasowicz, M., Morice, C., Ferrari, P., Callebert, J., & Versaux-Botteri, C. (2002). Long-term effects of light damage on the retina of albino and pigmented rats. *Investigative ophthalmology & visual science*, 43(3), 813–820.

Werling, D. M., & Geschwind, D. H. (2013). Sex differences in autism spectrum disorders, *Current Opinion in Neurology,* 26(2), 146-153.

Xu, X. L., Pan, C., Hu, J. X., Liu, X. T., Li, Y. F., Wang, H., Chen, Y. B., Dong, H. Y., Dai, T. J., & Xu, L. C. (2012). Effects of isoflurane inhalation on the male reproductive system in rats. *Environmental toxicology and pharmacology*, 34(3), 688–693.

Yeh, S., Miyamoto, H., Shima, H., & Chang, C. (1998). From estrogen to androgen receptor: a new pathway for sex hormones in prostate. *Proceedings of the National Academy of Sciences of the United States of America,* 95(10), 5527–5532.

Zorumski, C. F., Paul, S. M., Izumi, Y., Covey, D. F., & Mennerick, S. (2013). Neurosteroids, stress and depression: potential therapeutic opportunities. *Neuroscience and biobehavioral reviews*, 37(1), 109–122.