EFFECTS OF GONADECTOMY AND INHIBITION OF ANDROGEN SYNTHESIS ON BEHAVIOURAL FLEXIBILITY IN MALE RATS

by

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ABSTRACT

Androgens regulate sexual and aggressive behaviour in males. However, little attention has focused on the effects of androgens on executive function. Androgens are produced in the gonads but are also produced in the brain, which might be important when systemic androgen levels are low. Here, we examined the effects of gonadectomy (GDX) and/or an androgen synthesis inhibitor (abiraterone acetate, ABI) on different forms of behavioural flexibility in adult male Long-Evans rats. Rats received either GDX or Sham surgeries and then were housed for 5 weeks, to allow for upregulation of local androgen synthesis after GDX. Five days prior to the commencement of behavioural training, rats received daily treatments of either Vehicle or ABI (40 mg/kg, p.o.), an androgen synthesis inhibitor that crosses the blood-brain barrier. Behavioural flexibility was assessed on an operant based strategy set-shifting task or a spatial reversal learning task. The strategy set-shifting task required rats to disengage from a previously correct (but now incorrect) visual-cue based discrimination strategy, and acquire and maintain a new egocentric spatial response strategy. During the set-shift to an egocentric response strategy, ABI treatment (but not GDX) caused an improvement in behavioural flexibility, by reducing the number of errors made before reaching criterion. In a separate group of rats trained on a reversal learning task, we found a similar effect, in that only ABI reduced perseverative-type errors during the reversal. During the set-shift and the response reversal, there were no effects of GDX, suggesting that GDX+Vehicle subjects maintain or upregulate neural androgen synthesis to maintain baseline flexibility. Using liquid chromatography tandem mass spectrometry, we measured testosterone (T) in the medial prefrontal cortex (mPFC) and the dorsomedial striatum (DMS). Neural T was only detectable in the Sham+Vehicle rats, suggesting that GDX+Vehicle rats may have neural T synthesis occurring in other brain regions important for behavioural flexibility. Taken together, these data suggest that neural T synthesis may serve to increase persistence of behaviour, which can in some instances suppress behavioural flexibility.

LAY SUMMARY

Androgens are important steroid hormones that affect many different behaviours, including sexual and aggressive behaviour. However, it is unclear how steroids affect higher order cognitive abilities like cognitive flexibility. Sex steroids like testosterone are typically produced in the gonads but more recently it has been shown that testosterone can be produced in the brain. Neurally-produced testosterone may become particularly important when the testosterone produced in the gonads is low. In this study, we used rats to look at cognitive flexibility and we wanted to see how testosterone produced in the brain might help maintain normal behavioural flexibility. We found that when we reduced testosterone produced in the brain, animals were more flexible and persisted less with old strategies. When we made gonadal testosterone low but left testosterone produced in the brain, these animals performed like animals with no treatments. This suggests that testosterone produced in the brain promotes persistence.

PREFACE

All experiments were conducted at the University of British Columbia (Vancouver Campus) and carried out by Ryan Tomm. The experimental design, concept formation, statistical analyses and writing were completed by Ryan Tomm with the guidance of Dr. Kiran Soma and Dr. Stan Floresco, who were the supervisory authors on this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-hydroxytryptamine; serotonin (5-HT)
Abiraterone acetate (ABI)
Corticosterone (CORT)
Dehydroepiandrosterone (DHEA)
Dehydroepiandrosterone Sulfate (DHEA-S)
Dopamine (DA)
Dorsomedial striatum (DMS)
Estradiol (E2)
Gonadectomy (GDX)
High-performance liquid chromatography (HPLC)
Hypothalamic-pituitary-gonadal (HPG) axis
Liquid chromatography tandem mass spectrometry (LC-MS/MS)
Medial prefrontal cortex (mPFC)
Mobile phase A (MPA)
Mobile phase B (MPB)
Multiple reaction monitoring (MRM)
Nucleus accumbens (NAc)
Per os; oral administration (p.o.)
Phosphate buffered saline (PBS)
Prefrontal Cortex (PFC)
Sham surgery (Sham)
Testosterone (T)
Ventral tegmental area (VTA)

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Finally, I would like to thank everyone in the Soma Lab and Floresco Lab that have given me support over all these years! You guys are all awesome!

This is dedicated to most important women in my life:

Audrey

Jean

Penny

Mercedes

Angelina

I wouldn't be here if it wasn't for your love and sacrifice

INTRODUCTION

Steroid hormones are potent signalling molecules that typically travel from where they are produced to distant target tissues to regulate different aspects of physiology. Steroids are produced in a variety of tissues from cholesterol and can be transformed by different enzymes into progestins, glucocorticoids, mineralocorticoids, androgens, and estrogens. Steroids can affect neurophysiology to regulate a variety of behavioural functions, including sexual behaviour, aggression, decision making, and executive function. Over the decades, steroid research has largely focused on elucidating the neural mechanisms of sexual behaviour and aggression, with less effort focusing on the neural mechanisms that underlie steroidal modulation of executive function. The focus of this thesis is the specific roles of the sex steroids testosterone (T) and estradiol (E2) in the regulation of executive function.

Executive function is an overarching term used for a variety of dissociable cognitive functions that are regulated mainly by the prefrontal cortex (PFC) in a fashion of top-down control over sub-cortical structures to affect behaviour (Robbins & Arnsten 2009). Interestingly, many of the different aspects of executive function are regulated by steroid hormones, including working memory, cognitive control, risk assessment, and behavioural flexibility (Andrew & Rogers 1972, Fader et al 1999, Janowsky et al 2000, Kritzer et al 2007, Kritzer et al 2001, Spritzer et al 2008, Thompson & Wright 1979, Wallin et al 2015, Wallin & Wood 2015). Therefore, understanding the modulatory role of steroid hormones on PFC-mediated executive function can give us important insight into the physiology of psychiatric disorders that are differentially represented between the sexes, cognitive decline seen during aging, or any other disorder that occurs when systemic or neural steroids are perturbed compared to the normal population.

Influence of sex steroids on executive function

The modulation by sex steroids of working memory, one aspect of executive function, has been extensively studied in both human and animal models. Working memory is the ability to maintain and manipulate information, typically over a short period of time to flexibly guide future behaviour (Goldman-Rakic & Friedman 1991). This PFC-mediated form of executive function shows robust impairments in males with low levels of the sex steroid T, such as during male aging (Janowsky et al 2000), and in prostate cancer patients undergoing androgen deprivation therapy (Beer et al 2006). Furthermore, hormone replacement therapy of E2 in postmenopausal females improves deficits observed on cognitive tasks relying on working memory (Duff & Hampson 2000).

In animal models, we are afforded with the opportunity to manipulate sex steroids in ways that help us mimic what we find in the human clinical populations. Interestingly, when the main source of sex steroid production is removed from either male or female rats via gonadectomy (GDX), impairments in working memory are analogous to those seen in the human clinical populations discussed above (Fader et al 1998, Fader et al 1999, Gibbs & Johnson 2008, Kritzer et al 2007, Sandstrom et al 2006, Spritzer et al 2008). For example, GDX male rats are impaired compared to intact controls on a spatial working memory task, and this effect is reversed if T is administered after GDX (Sandstrom et al 2006). Likewise, female rats receiving E2 perform better on a working memory task compared to female rats receiving vehicle (Fader et al 1999). Thus, it appears that this form of PFC-mediated executive function is differentially mediated in males and females by T or E2 respectively (Fader et al 1999, Sandstrom et al 2006). Although our understanding of the regulation of sex steroids on working memory is becoming clearer, there are other aspects of executive function that remain to be explored.

Behavioural flexibility is another aspect of executive function regulated by the PFC that is influenced by sex steroids, but this topic has remained relatively unexplored. Behavioural flexibility is the ability to adapt behavioural responses when environmental circumstances are changing. Behavioural flexibility has been described as a hierarchical process, regulated mainly by distinct but sometimes overlapping neural networks (Floresco et al 2009). Reversal learning is one form of behavioural flexibility that requires organisms first learn a stimulus-response association between many possible options within the same stimulus dimension, only one of which is reinforced. Next, when reinforcement contingencies are changed, organisms must disengage from the previous stimulus-response association and sample the other stimuli to make new reinforcement associations (e.g., "This key does not unlock the door anymore, so maybe I should try my other key"). Strategy set-shifting is another form of behavioural flexibility that requires organisms to first learn a stimulus-response strategy for reinforcement. Next, when reinforcement contingencies are changed (i.e., the rule changes), organisms must disengage from the previous stimulus-response strategy and explore new stimulus-response strategies (e.g., "This key does not unlock the door anymore, so maybe I should try to climb through the window"). There are some circumstances when persistence for a strategy or rule might be adaptive for an organism, such as when reinforcement contingencies are probabilistic or non-deterministic. This is the nature of overcoming adverse circumstances to persist despite losses or hardships. However, in many cases the inability to disengage by persisting in a previously correct but now incorrect strategy could be maladaptive for an organism.

Studies looking at the influence of T on behavioural flexibility were first described in the 1970's in chicks. Here, researchers found that male chicks given T would persist with pecking for a particular food in specific locations in a dose-dependent manner compared to male chicks that

did not receive T, despite other food options being available. Furthermore, female chicks receiving the same doses of T did not show such persistence in food selection and search behaviour compared to controls (Andrew & Rogers 1972). Similar effects of T on food search behaviour and perseverative tendencies have been observed in male mice and rats (Archer 1977, Neese & Schantz 2012, Spritzer et al 2011, Thompson & Wright 1979, van Hest et al 1989). For example, in an experiment utilizing adult male rats, researchers found that intact and GDX rats given T had higher lose-stay tendencies compared to GDX rats in a delayed spatial alternation task (Neese & Schantz 2012). In another experiment, researchers found that male rats given T needed more trials to reach criterion performance, whereas male rats receiving cyproterone acetate (an androgen receptor antagonist/androgen synthesis inhibitor) needed fewer trials to reach criterion performance, compared to male rats receiving vehicle using a strategy set-shifting task in operant chambers (Thompson & Wright 1979). More recent evidence of T regulation of behavioural flexibility comes from rodent studies of anabolic-androgenic steroid abuse. Here, researchers found that intact young male rats given chronic supraphysiological doses of T during adolescence needed more trials to reach criterion performance on a reversal learning and strategy set-shifting task (Wallin & Wood 2015). Furthermore, effects of T on persistence are observed in human males after competition loss, where males that had increased levels of T after a loss were more likely to compete again compared to males that had decreased levels of T after a loss (Mehta & Josephs 2006, Welker & Carré 2015). Thus, it appears that higher T levels in males decrease flexible behaviours, whereas lower levels of T increase flexible behaviours through alterations in persistence.

Gonadal and neural sex steroid production

Traditionally, sex steroid production has been thought to be regulated by the hypothalamic-pituitary-gonadal (HPG) axis, where gonadotropins are secreted by the hypothalamus to stimulate release of hormones from the pituitary into the bloodstream (luteinizing hormone and follicle-stimulating hormone) in which they travel to the gonads to stimulate sex steroid production. Sex steroids are then released into the bloodstream and travel throughout the body to have their effects on different tissues and further release is regulated through a complex system of positive and negative feedback. However, this model of sex steroid regulation is slowly being expanded with increasing evidence that steroids can be produced locally in a variety of tissues that were originally thought to be the targets of steroid action (Fokidis et al 2015, Hojo et al 2004, Taves et al 2017, Taves et al 2011a). The exact mechanisms of how local sex steroid synthesis is increased relative to traditional gonadal production remains to be determined; however, it is suggested that local synthesis might increase when systemic levels are low in order to maintain normal behavioural and physiological functions (Fokidis et al 2015, Soma et al 2015).

The notion of "neurosteroids" was first introduced in the 1980's by Dr. Étienne-Émile Baulieu and collogues when they observed that the rat brain contained large amounts of DHEA-S that was independent of the levels found through gonadal or adrenal production (Corpechot et al 1981). Since this time there are many lines of converging evidence to show that sex steroids can be produced locally in a variety of tissues independent of traditional gonadal synthesis. For example, in prostate cancer, tumor growth is driven by androgen action within the tumor, leading to castration as a treatment of mitigating the deleterious effects of androgens within the tumor (Fokidis et al 2015, Huggins & Hodges 1941, Sun et al 2016). Castration resistant prostate cancer occurs when the tumor begins to grow even in the absence of gonadal production of androgens.

One experiment, looking at the mechanisms of this phenomenon in rodents, transplanted mice with prostate cancer cells and measured androgen levels in the tumor before GDX, and at 8 days or 35 days after GDX. Interestingly, it was found that the tumor contained elevated levels of androgens 35 days after GDX and that the tumor contained the enzymes necessary for androgen production (Locke et al 2008). This suggests that after time, the tumor can produce its own androgens to facilitate growth when systemic androgen levels are absent.

The brain also contains mRNA for all the enzymes involved in steroid production from cholesterol (Hojo et al 2004, Hojo et al 2011, Kimoto et al 2010, Mellon & Deschepper 1993). Furthermore, the brain is abundant in cholesterol, containing about 20% of the body's total cholesterol (Orth & Bellosta 2012). One tissue culture experiment looking at different types of steroidogenic cells in the cerebral cortex of neonatal rat brains found that astrocytes and neurons were capable of producing more of the androgen DHEA when steroid precursors were added to the tissue culture (Zwain & Yen 1999). Indeed, the molecular machinery and materials are available in the brain to produce neurosteroids; however, more importantly, the behavioural functions of neurosteroids are relatively unexplored.

Evidence for neurosteroids influence on behaviour

Avian studies are some of the first to suggest a role for neurosteroids in modulating behaviour. Song sparrows are small birds found in North America that are highly aggressive when competing conspecifics intrude into their territories. This aggressive behaviour has been shown to be regulated by the sex steroids T and E2 in male songbirds (Soma 2006, Soma et al 2000, Soma & Wingfield 2001a, Soma & Wingfield 2001b). Interestingly, this aggressive behaviour is found year-round, in both the breeding season (when circulating sex steroids are high) and in the non-breeding season (when circulating sex steroids are low) (Soma & Wingfield 2001b). Thus, during

the non-breeding season when circulating sex steroids are non-detectable, male song sparrow aggression is still maintained, which led some researchers to hypothesize that neurosteroids might be mediating these effects (Wingfield et al 2001). In one experiment, male song sparrows were treated with vehicle or the aromatase inhibitor fadrozole in the non-breeding season and aggressive behaviour was elicited using a simulated territorial intrusion. It was found that aggression in the male song sparrows receiving fadrozole systemically was reduced compared to song sparrows receiving vehicle and that aggressive behaviour was restored when co-administered with E2 (Soma et al 2000). This provided indirect evidence that sex steroids might be synthesized de novo from cholesterol in the brain, or regulated locally from other precursor hormones in the blood, to help maintain aggressive behaviour in male song sparrows during the non-breeding season. Research on the local synthesis of sex steroids in the brain and the effects on behaviour remain limited. This has led to the goal of the current study, to elucidate the behavioural effects of neurosteroids in mammals, which may have implications for changes in executive function observed during human male aging or those seen during treatment of castration resistant prostate cancer.

The present study looks to address the gap in how neurosteroids may regulate behavioural flexibility when systemic steroid levels are low. To do this, we are using multiple assays of the different components of behavioural flexibility in young male rats (strategy set-shifting, and spatial reversal learning), which are dependent on the PFC (and sex steroid signalling). Here we utilized a 2 x 2 design (Fig. 1), with animals receiving either Sham or GDX surgery, and Vehicle or the androgen synthesis inhibitor abiraterone acetate (ABI), a drug used to treat men with castration resistant prostate cancer. Our hypothesis is that GDX animals will have increased synthesis of neurosteroids to keep behavioural flexibility intact and that animals receiving ABI, which inhibits neural androgen synthesis, will have improved behavioural flexibility. To assess this, we will

measure a panel of steroids in the blood and in multiple brain regions that are known to regulate behavioural flexibility, including the medial prefrontal cortex (mPFC). To our knowledge, we are the first research group to assess the behavioural effects of neuroT in mammals.

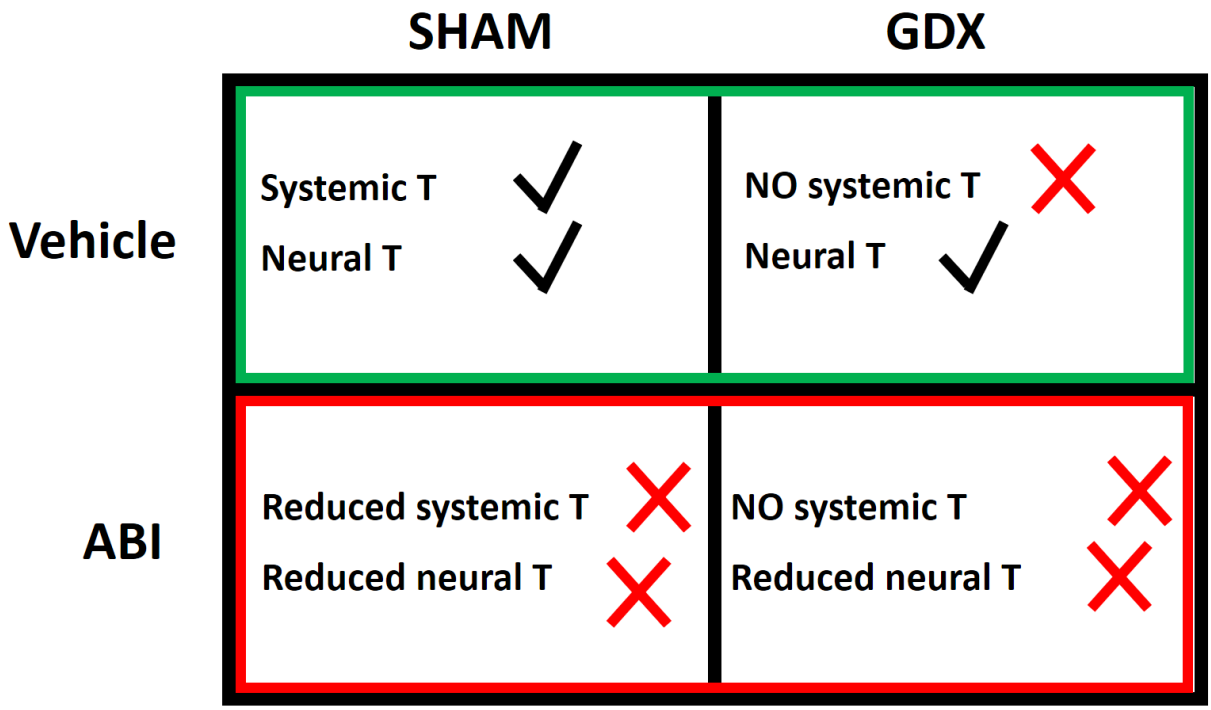

Fig. 1. Experimental design. Vertical columns represent surgery and horizontal rows represent drug. Our hypothesis is that GDX+Vehicle animals will have neural T even in the absence of gonadal production. The green box represents animals that will have intact neural T and should perform similarly on tests of behavioural flexibility. The red box represents animals that will have reduced neural T and should show different behavioural patterns compared to animals with intact neural T.

MATERIALS AND METHODS

Animals

Cohorts of male Long Evans rats were obtained from Charles River (Raleigh/Montreal) weighing 250-300g. In the first week after arrival, each cohort of animals was group housed (4 per cage) in clear polycarbonate cages (20”D x 16”W x 8.5”H) with stainless steel lids, and given *ad libitum* access to Rat Diet 5012 (PMI Feeds Inc.) and to water. Each cage had aspen chip bedding (Nepco), paper towel for nesting, and two PVC pipes for environmental enrichment. After the first week, animals were randomly assigned to each condition and received Sham or GDX surgery (see Gonadectomy Surgery), after which they were single housed in clear polycarbonate cages (19”D x 10.5”W x 8”H) with stainless steel lids, one PVC pipe, and paper towel. For 3 weeks, all animals were continued on *ad libitum* access to food and water, after which they were food restricted to 85-90% of their free-feeding weight for the remainder of the experiment (See Fig. 2 for timeline).

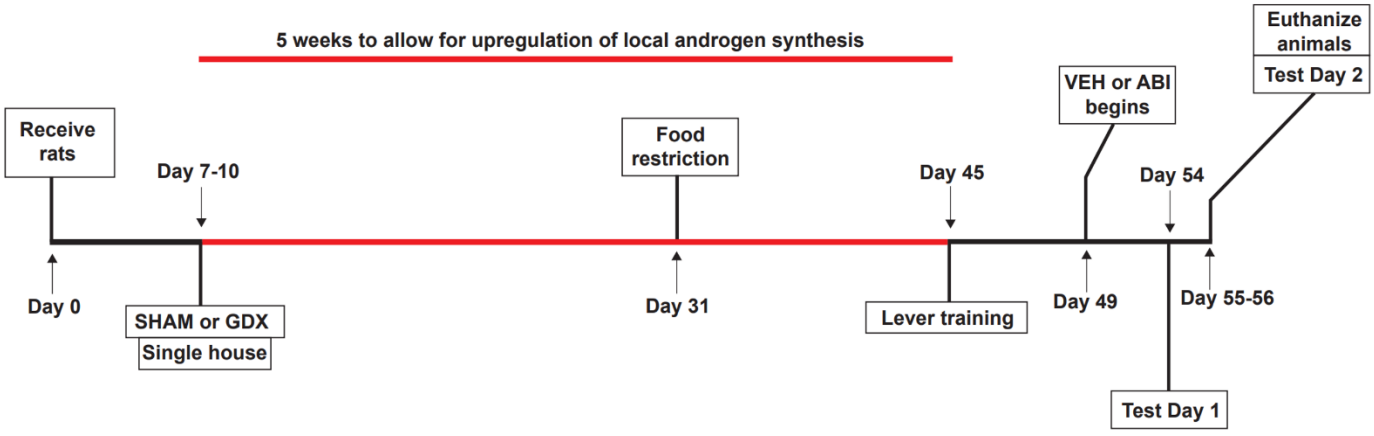

Fig. 2. Timeline for experiment 1 and 2. Red lines indicate the amount of time to allow for upregulation of local synthesis in the brain. For the strategy set-shifting experiment, test day 1 was the visual-cue discrimination and test day 2 was the shift to response discrimination. For the spatial reversal learning experiment, test day 1 was the initial response discrimination and test day 2 was the response reversal discrimination. Animals in both the strategy set-shifting experiment and the spatial reversal learning experiment were euthanized the day after completion of operant testing.

Animals were maintained on a 12hr light/dark cycle (lights on at 12pm), with the colony room temperature at 21˚C and a relative humidity of 40-50%.

Gonadectomy Surgery

All surgeries were carried out under aseptic conditions with isoflurane anesthesia approximately 5 weeks before behavioural training began. This time frame was decided on from prostate cancer research, which shows that tumors will begin to produce their own androgens about 5 weeks after castration (Locke et al 2008). Once animals reached a surgical plane of anesthesia, a local injection of bupivacaine (2.5mg/ml) was made to the site of the incision and the scrotum and tunic muscle was cut to expose the testes for removal. For Sham surgery, the tunic and scrotum were closed using sterile sutures (4-0, absorbable). For GDX surgery, the vas deferens were bilaterally ligated and the testes were removed before suturing. Animals were monitored closely after surgery and received subcutaneous injections of buprenorphine (0.03mg/kg) and anafen (5mg/kg) to help alleviate post-surgical discomfort.

Drug administration

Animals were habituated to and fed a 50/50 mixture of ground rat chow and peanut butter (Kraft, smooth) which was used as the Vehicle for drug administration. For drugs groups, the androgen synthesis inhibitor ABI (Fig. 3A; MedChemExpress; Princeton, NJ) was mixed with the vehicle using a food processor (Presidents Choice Mini Chop) and dosing was calculated using the average of each group's body weight. Beginning 5 days prior to the first behavioural test day, animals were fed (p.o., 4hr before testing) 1mg of Vehicle or ABI mixture (40mg/kg) daily for the remainder of the experiment. This dose of ABI given i.p. has been shown to have behavioural effect in rats (Frau et al 2014) and effectively reduces testosterone levels in the serum of rats and

mice (Barrie et al 1994, Duc et al 2003, Haidar et al 2003). Furthermore, after oral administration, ABI reaches peak levels in a variety of tissues within 4 hr (European Medicines Agency, 2011). We confirmed these findings in a pilot study using intact male Long Evans rats (~400-500g) that received either Vehicle or ABI (40mg/kg) as described above for 4 days. Here we found that ABI was effective in reducing T levels in serum and brain tissue compared to Vehicle animals (unpublished results). Furthermore, ABI was able to cross the blood brain barrier as measured in serum and brain tissue of animals receiving ABI (unpublished results).

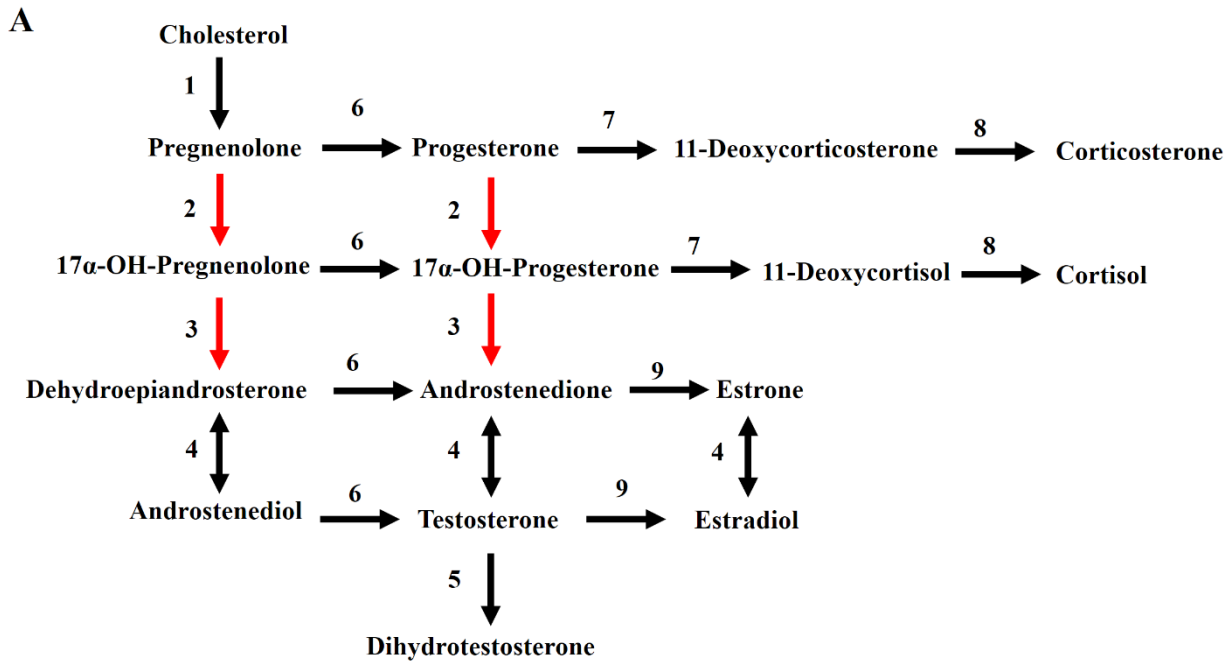

Fig. 3. Steroid synthesis pathway. Numbers indicate proteins and enzymes: (1) StAR, (2) CYP17A1, (3) CYP17A1, (4) 17β-HSD, (5) 5α-reductase, (6) 3β-HSD, (7) 21-hydroxylase, (8) 11β-hydroxylase, (9) aromatase. Red arrows indicate conversions inhibited by abiraterone acetate.

Apparatus

For all experiments, testing was done with 16 operant chambers (30.5 × 24 × 21 cm; Med Associates, St Albans, VT, United States of America). All chambers were within sound attenuating boxes and ventilated with fans that helped mitigate outside noise. At the back wall of each chamber was a 100mA house light that illuminated the box. Located in the center of the front wall was a food trough where food reinforcement (45 mg; Bioserv, Frenchtown, NJ, United States of America) was delivered by a pellet dispenser. Two retractable levers were located one on each side of the food trough with one light above each. Infrared photobeams were mounted in the chamber to measure the locomotor activity. The operant chambers were connected to MedPC computer software which operated the program and stored the data.

Lever press training

For both experiments, procedures were adapted from Floresco, Block & Tse (2008) and Butts, Floresco & Phillips (2013). All testing was done during the middle of the light cycle, and the day before behavioural training began animals were familiarized with the food reinforcement and given ~ 20 sugar pellets in their home cage. On the first day, animals were habituated to the chambers for 30 min, during which food reward was dispensed intermittently. Following habituation, animals were trained under a fixed-ratio 1 schedule to press each lever (one at time; counterbalanced) to a criterion of 60 lever presses. If animals made 60 lever presses in 30 minutes they were switched to the opposite lever on the next training day. If criterion was not reached, animals were re-trained on the same lever until criterion was met. After criterion was reached on both levers, the animals were moved onto retractable lever press training and drug administration was started. Here, animals were trained over 90 trials to press an extended lever within a limited time for food reward. Before each trial the house light was off and both levers were retracted.

Every 20 seconds a trial was initiated with the house light turning on and one of the two levers was extended. Animals had 10 seconds to press the extended lever or the trial was counted as an omission and the house light was terminated. On the 5th day of retractable lever-press training, animals with fewer than 10 omissions in 90 trials were immediately tested for lever side preference.

Side Preference Test

For the first time, animals had to select between both levers. In the first trial, a selection of either lever would result in food reward and levers were retracted. Next, levers were re-extended and the animal would have to select the opposite lever to receive a food reward. If the animal did not select the opposite lever, every 20 seconds the levers were re-extended, and this process continued until animals choose the opposite lever (1 trial). The program tracked the first choice over a total of 7 trials, and from this data the side preference of each animal was determined.

Experiment 1: Strategy Set-Shifting

Visual-cue Discrimination

This test day consisted of 30-150 trials with an inter-trial interval of 20 seconds. Every trial began with one of the two stimulus lights (pseudo-randomly, either left or right) turning on. After 3 seconds, both levers extended and the house light was turned on. If animals did not make a selection within 10 seconds, the levers retracted, the lights extinguished and the trial was counted as an omission. To receive reward, animals had to select the lever with the light above it (ie. visual-cue discrimination; Fig. 5A left panel). The program was terminated if the animal completed a criterion of 10 consecutive correct choices (after a minimum of 30 trials), or after a maximum of 150 trials. If animals did not complete 10 consecutive correct choices in 150 trials, one more day of visual-cue discrimination training was given.

Shift to Response Discrimination

This test day began with 20 reminder trials of the visual-cue discrimination. After 20 trials, the rule shifted so that animals must select the lever opposite of the individual animals' side preference. Thus, animals must ignore the visual-cue rule and shift strategies to an "egocentric" response rule (ie. always respond to the left lever, irrespective of where the stimulus light is located; Fig. 5A right panel). The program terminated if the animal completed a criterion of 10 consecutive correct choices or after 160 trials (after the shift).

During the shift, animals could make 3 different types of errors: perseverative, regressive, and never-reinforced. An animal made a perseverative error if it selected the lever with the light illuminated above it, on trials that required responding on the opposite lever (possible on half of the trials). Thus, the animals would persist on the old "visual-strategy" of a light-cue predicting reward, instead of changing to a new "response-strategy" that is now predictive of reward (ie. responding to the lever opposite of their side bias). Perseverative responding was scored when an animal made this type of error on 6 or more trials in a block of 8 trials where this type of error was possible. Perseverative errors are indicative of an animals' ability to disengage from a previously correct but now incorrect strategy.

However, if animals made perseverative-type errors on 5 or less trials in each block of 8 trials, subsequent errors were then scored as a regressive error. Thus, although the animal may show an understanding of the new response-strategy, shown by decreased visual-strategy performance, the animal still had a tendency (from time to time) to "regress" back to the old visual rule. Regressive errors are indicative of animals' ability to maintain a new strategy.

Finally, a never-reinforced error was scored when animals selected a lever that was not illuminated with a light, nor the lever opposite to the animals' side-preference. For example, before the shift the animal is only reinforced on the lever with the light illuminated above it (could be left or right lever), after the shift (assuming a right lever side preference) the animal is only reinforced on the left lever. Thus, in this example a never-reinforced error would be scored if the animal pressed the right lever on trials when the stimulus light was not illuminated above it, which is possible in half of the trials. Therefore, the animal is ignoring both the old visual-strategy and the new response-strategy, by making a response it has never been reinforced on. Never-reinforced errors are indicative of animals' ability to parse out ineffective strategies.

Experiment 2: Spatial Reversal Learning

Response Discrimination

Experiment 2 followed the same behavioural training procedures, inter-trial interval, and omission criterion as experiment 1 but was tested in a separate cohort of animals. The only difference between this task and the strategy set-shifting task is the light is never predictive of reward and shifts are within the same stimulus dimension. On the first test day, rather than select the lever with the light above it to receive reward, animals must select the lever opposite to their side preference, irrespective of light position. Thus, this task required animals to adopt only an egocentric response based strategy if it were to successfully receive reward. The program was terminated if the animal completed a criterion of 10 consecutive correct choices (after a minimum of 30 trials), or after a maximum of 150 trials. If animals did not complete 10 consecutive correct choices in 150 trials, one more day of response discrimination training was given.

Response Reversal Discrimination

This test day began with 20 reminder trials of the initial response discrimination. After 20 trials, the rule reversed so that animals must select the lever opposite to the initial response rule on day 1. For example, if animals had to respond on the left lever during the initial response discrimination on day 1, then during the reversal, animals had to select the right lever for reward (or vice versa depending on side preference). During both test days, the lever lights were not predictive of reward and acted as a distractor. The program terminated if the animal completed a criterion of 10 consecutive correct choices or after 160 trials (after the reversal).

In this experiment, animals could make 2 different types of errors during the response reversal: perseverative, and regressive. A perseverative error was scored if an animal made an incorrect response by selecting the lever that was rewarded during the initial response discrimination. Thus, the animals persisted on the old response rule that predicted reward, instead of shifting to the new response rule that is now predictive of reward (ie. responding on the opposite lever than test day 1). Perseverative responding was scored when an animal made this type of error on 10 or more trials in a block of 16 trials. All other errors were scored as regressive.

Tissue Collection

After behavioural testing, animals in both experiments were returned to their home cage and fed. The following day, animals were treated as if they were undergoing one more day of testing. Animals received Vehicle or ABI at the usual time of day and were euthanized at the time when they would have been tested in the operant chambers.

At the time of euthanasia, animals were approximately 4 months old (post-natal day 115-120). Animals were transferred one at a time (in their home cage) to a separate room where all

anesthesia was performed. Animals were rapidly anesthetized with isoflurane (Pharmaceutical Partners of Canada Inc.). Once deeply anesthetized, animals were transferred to another room and were euthanized via rapid decapitation (for steroid analysis) or transcardial perfusion (for immunohistochemistry). In both experiments, half of the animals from each experimental group was randomly assigned to rapid decapitation or transcardial perfusion. All animals were euthanized under 3 min (from the start of isoflurane to time of blood collection). These procedures were used to prevent the corticosterone (CORT) response from affecting androgen levels in the blood and brain (Taves et al 2011b).

Animals undergoing rapid decapitation were allocated to measure steroid levels in the blood and brain. After decapitation with a guillotine, trunk blood was collected and the serum was separated via centrifugation for 10 min at 16100×g and stored in -80˚C. Brains were removed, rapidly frozen in powdered dry ice within 6 min from decapitation and immediately stored at -80˚C. Brains were coronally sectioned in a cryostat (-12ºC) from rostral to caudal at a thickness of 300µm. Brain sections were then placed on glass microscope slides for storage (-80˚C).

Animals undergoing transcardial perfusion were used to measure steroid levels in the blood; however, here brains were fixed for later immunohistochemical analysis of different dopamine (DA) signalling proteins. Before transcardial perfusion, the heart was exposed and animals were maintained on isoflurane while blood from the right atrium was collected using a syringe. The serum was separated via centrifugation for 10 min at 16100×g and stored in -80˚C. Once blood was collected, a butterfly needle was inserted into the left ventricle and animals were perfused with 50mL of room temperature phosphate buffered saline (PBS; 0.1 M) and then 250mL of freshly-made room temperature 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde in PBS for 4 hr at room temperature. Then brains were washed in

PBS for 1.5 hr (3x30min) before cryo-protection in 30% sucrose for 48hr at 4 ˚C. After brains sunk to the bottom of the sucrose solution, brains were removed and stored at -80˚C. Brains for animals undergoing this procedure, were coronally sectioned in a cryostat (-20ºC) from rostral to caudal at a thickness of 40µm. Brain sections were then placed in anti-freeze (1% wt/vol polyvinylpyrollidone, 30%wt/vol sucrose, and 30% vol/vol ethylene glycol in PBS) and stored at -20˚C until brains were ready for immunohistochemistry. For the purposes of this thesis, data from immunohistochemical analysis will not be included because it is still being collected.

Steroid Extraction and Measurement

Brain Micro-dissection

Micro-dissection of the brain using the Palkovits punch method (Palkovits 1973, Taves et al 2011b) allows for collection of discrete brain regions involved in behavioural flexibility. Here, we wanted to measure local steroid concentrations in the prelimbic and infralimbic portions of the medial prefrontal cortex (mPFC) and the dorsomedial striatum (DMS), two brain regions implicated in the regulation of behavioural flexibility (Birrell & Brown 2000, Floresco et al 2006b, Ragozzino et al 1999a, Ragozzino et al 1999b). Brain sections on glass microscope slides were placed on a stainless-steel platform (6 inch diameter) cooled with powdered dry ice. Regions were punched bilaterally using a 1mm diameter tissue punching kit (Integra Miltex; York, PA) and stored in 2mL microcentrifuge tubes (Sarstedt; Newton, USA) at -80˚C. Brain punches 1mm in diameter at 300µm thickness have been shown to weigh on average 0.245mg (protein content 0.02mg) with very little variation (Taves et al 2011b). The mPFC yielded 2.94 ± 0.04 mg or 2.91± 0.04 mg and the DMS yielded 3.04 ± 0.04 mg or 2.95 ± 0.03 mg of wet tissue weight for the strategy set-shifting and spatial reversal learning animals, respectively. Brain regions were located using Paxinos and Watson's (2007) "Rat Brain in Stereotaxic Coordinates" (See Fig. 4. for

coordinates and location of punches) and sections were Nissl stained to verify locations of punches. If punches fell outside of their respective coordinates, they were excluded from analysis.

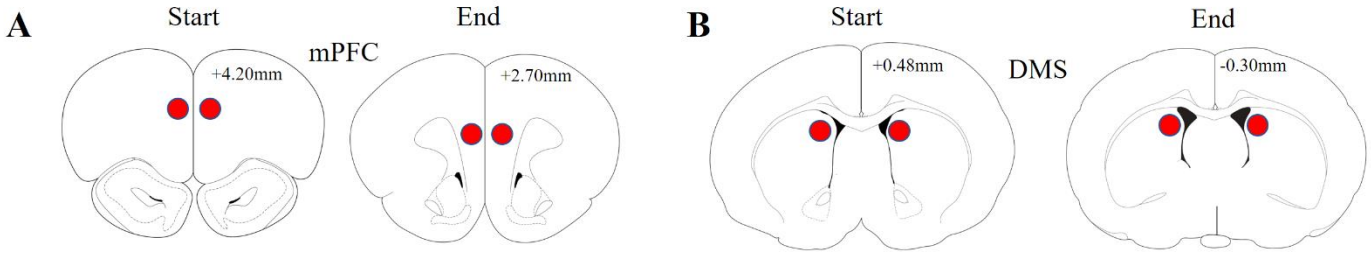

Fig. 4. Schematic of the rat brain showing different locations for bilateral microdissections in red circles (1mm diameter) using the Palkovits punch method. (A) Medial Prefrontal Cortex (mPFC); (B) Dorsal Medial Striatum (DMS). The left side of each panel indicates the starting location for tissue collection relative to Bregma for each brain region. The right side of each panel indicates the ending location for tissue collection relative to Bregma for each brain region.

Homogenization and Solid Phase Extraction

Steroid extraction and measurement was adapted from Korol et al (2017) with minor adjustments. Steroids were extracted from brain punches and blood serum (2 µL) with 500 µL of acetonitrile with 0.01% formic acid. Next, deuterated internal standards (50 µL) in 50% HPLC-grade methanol in dH2O was added to each sample (testosterone-d5, corticosterone-d8, progesterone-d9, 17β-estradiol-d4, and DHEA-d6). Each micro-centrifuge tube contained 3 zirconium ceramic oxide beads (1.4 mm diameter) and samples were homogenized with a bead mill at 5m/s for 30 sec (Omni International Inc; Kennesaw, GA). Samples were stored in -20ºC for at least one hour to precipitate proteins. Next, samples were centrifuged for 5 min at 16100×g and 400 µL of the supernatant was transferred to glass vials. The left-over samples were then resuspended in another 400 µL of acetonitrile with 0.01% formic acid and vortexed for 5 sec.

Samples were re-centrifuged for 5 min at the same speed and another 400 µL of the supernatant was added to the previously extracted samples in the corresponding glass vials. Once this was complete, samples were dried in a vacuum centrifuge (ThermoElectron SPD111V) at 60ºC for 45 min and stored in -20ºC until resuspension. Blanks and 11 standard curve samples were prepared using the same process (standard curve range; T, CORT, Progesterone, E2 [0.2-1,000 pg/tube]; DHEA [4-10,000 pg/tube]).

Samples were resuspended in 500 µL of HPLC-grade methanol and vortexed for 10 sec. Steroids were then extracted via solid phase extraction in C18 columns (Agilent, cat. 12113045). First, the columns were primed in order with 3 mL of HPLC-grade hexane, 3 mL of HPLC-grade acetone, and 3 mL of HPLC-grade methanol. Resuspended samples were then loaded into the columns and the eluate was collected. Then 2mL of HPLC-grade methanol was used to elute the remaining sample, which was also collected. Finally, samples were dried in a vacuum centrifuge at 60ºC for 1.5 hr and stored at -20ºC until measurement with liquid chromatography tandem mass spectrometry (LC-MS/MS).

LC-MS/MS Measurement of Steroids

Samples were resuspended in 50 µL of 50% HPLC-grade methanol in MilliQ water and vortexed for 2 sec before being transferred into a refrigerated autoinjector (15ºC). Next, 45 µL of the resuspended sample was injected into a Nexera X2 system (Shimadzu Corp; Kyoto, Japan). The sample was then passed through a Poroshell 120 HPH C18 guard column (2.1mm) and separated in a Poroshell 120HPH C18 column (2.1 x 50 mm; 2.7 µm; at 40ºC) using 0.1 mM ammonium fluoride in MilliQ water for the mobile phase A (MPA) and HPLC-grade methanol for the mobile phase B (MPB) at a flow rate of 400 µL/min. MPB was at 10% for 36 sec during loading, after which the MPB gradient profile shifted to 40%, then to 70% in 5.4 min, and ended

in a column wash at 98% for 1.6 min. Between each sample, the MPB was returned to starting conditions for 1.3 min with a total run time of 9 min for each sample. The needle was rinsed externally with 100% isopropanol between each sample. Samples were detected with multiple reaction monitoring (MRM), with two MRM transitions for T, CORT, Progesterone, E2, and DHEA, and one MRM transition for internal standards. Steroid concentrations were acquired on an AB Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (AB Sciex LLC, Framingham, MA) in a positive electrospray ionization mode for T, CORT, Progesterone, and DHEA, and in a negative electrospray ionization mode for E2.

Statistical Analysis

For the *strategy set-shifting task,* the primary dependent variable of interest was the number of errors to criterion, in both the initial learning of the visual-cue discrimination and the shift to response discrimination. We averaged the amount of errors from each group and used a two-way ANOVA (Surgery x Drug) to determine performance. The number of trials before reaching criterion, locomotor activity, response latencies, and omissions were also analyzed in this way. The number of perseverative, regressive, and never-reinforced errors were analyzed using a three-way between-within ANOVA, with Surgery and Drug as the between-subject factors and Error Type as the within-subject factor.

For the *spatial reversal learning task,* the primary dependent variable of interest was the number of errors to criterion, in both the initial learning of the response discrimination and the response-reversal discrimination. We averaged the amount of errors from each group and used a two-way ANOVA (Surgery x Drug) to determine performance. The number of trials before reaching criterion, locomotor activity, response latencies, and omissions were also analyzed in this way. The number of perseverative, and regressive errors were analyzed using a three-way between-

within ANOVA, with Surgery and Drug as the between-subject factors and Error Type as the within-subject factor.

In both experiments, we wanted to determine differences in a panel of serum and brain steroid levels. Here, we used a two-way ANOVA (Surgery x Drug) to determine group differences in serum and brain steroid levels. When values are provided in the text, the mean and standard error of the mean (±SEM) are included. All statistical analysis was done using SPSS and used a $p \leq .05$ for statistical significance.

RESULTS

Abiraterone acetate improves strategy set-shifting performance

Day 1: Visual-cue discrimination

Here we wanted to determine whether GDX or inhibition of androgen synthesis would affect visual-cue discrimination learning on a strategy set-shifting task. One animal from the Sham+Vehicle group was euthanized prior to testing due to infection from surgery and was removed from behavioural analysis. There were no main effects of Surgery ($F(1,75)= 0.44$, $p>.50$) or Drug ($F(1,75)= 1.07$, $p=.30$) on the number of trials to criterion and no main effects of Surgery ($F(1,75)= 0.17$, $p>.50$) or Drug ($F(1,75)= 2.48$, $p =.12$) on the number of errors to criterion. However, trials to criterion and errors to criterion for the initial visual-cue discrimination were qualified by significant or near significant Surgery × Drug interactions ($F(1,75)= 5.55, 3.54$, $p<.05$, $p=.06$, respectively). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the ABI-treated groups for trials to criterion ($F(1,75)=4.62$, $p <.05$) and near significant effect for errors to criterion ($F(1,75)=2.67$, $p =.10$). Sham+ABI subjects needed more trials and trended towards making more errors before reaching criterion compared to GDX+ABI subjects in the visual-cue discrimination. Furthermore, there was a significant simple main effect of Drug in the GDX groups for trials to criterion ($F(1,75)=5.83$, $p <.05$; Table 1) and errors to criterion ($F(1,75)=6.05$, $p <.05$; Fig. 5B). GDX+Vehicle subjects needed more trials and committed more errors before reaching criterion compared to GDX+ABI subjects in the initial visual-cue discrimination.

Day 2: Shift to response discrimination

Here we wanted to determine whether GDX or inhibition of androgen synthesis would affect behavioural flexibility through performance on a shift to response discrimination. First, we wanted to determine whether any effect would be driven by the ability to remember the initial visual-cue discrimination. Analysis from these data revealed no main effect of Surgery ($F(1,75)= 0.30$, $p>.50$) or Drug ($F(1,75)= 0.68$, $p=.41$) on the ability to remember the initial visual-cue discrimination. However, this analysis was qualified with a significant Surgery × Drug interaction ($F(1,75)= 5.27$, $p<.05$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the Vehicle-treated groups for memory of the initial visual-cue discrimination ($F(1,75)= 4.20$, $p<.05$). Thus, GDX+Vehicle subjects remembered the visual-cue discrimination better than Sham+Vehicle subjects. Furthermore, there was a significant simple main effect of Drug in GDX-treated groups for memory of the initial visual-cue discrimination ($F(1,75)=5.10$, $p <.05$; Table 1). Thus, GDX+Vehicle subjects remembered the visual-cue discrimination better than GDX+ABI subjects.

Analysis of trials to criterion for the shift to response discrimination revealed no main effects of Surgery ($F(1,75)= 1.72$, $p=.19$) or Drug ($F(1,75)= 2.55$, $p=.12$) and no Surgery × Drug interaction ($F(1,75)= 0.02$, $p>.50$; Table 1). However, errors to criterion is a more sensitive measure of performance for the shift to response discrimination, so we used a between-within ANOVA design, with Surgery and Drug as the between-subject factors and Error Types as the within-subject factor. This analysis revealed no main effect of Surgery ($F(1,75)= 0.58$, $p>.50$) but found a significant main effect of Drug ($F(1,75)= 3.93$, $p=.05$) with no Surgery × Drug interaction ($F(1,75)= 0.01$, $p>.50$; Fig. 5C) for the between-subject factors. Furthermore, we found a significant effect of Error Type for the within-subject factor ($F(2,154)= 41.35$, $p<.001$), but no

significant Drug × Error Type interaction ($F(2,154)= 1.10$, $p=.33$; Fig 5D). Thus, irrespective of Surgery, ABI subjects made fewer errors before reaching criterion compared to Vehicle subjects during the shift to response discrimination. To investigate which error type was driving this effect, we further analyzed each error type separately. Analysis of the error types during the shift revealed a trend for a Drug effect on perseverative errors ($F(1,77)= 2.87$, $p=.095$) but no significant effect of Drug on regressive errors ($F(1,77)= 0.05$, $p>.50$) or never-reinforced errors ($F(1,77)= 1.84$, $p=.18$; Fig 5D). Thus, it appears that the effect of ABI in reducing the number of errors to criterion during the shift may be driven by a modest reduction in perseverative type errors.

To investigate whether these results were driven by motivation or activity levels we also analyzed response latencies, omissions and locomotor activity. Analysis from these data revealed a significant main effect of Surgery on response latencies ($F(1,75)= 13.46$, $p<.001$; Table 1), with GDX subjects taking longer to make a selection compared to Sham subjects. However, there were no significant main effects of Surgery ($F(1,75)= 0.09, 2.13$; $p>.15$) or Drug ($F(1,75)= 1.06, 1.87$; $p>.18$) on omissions or locomotor activity, respectively.

Furthermore, we wanted to analyze differences in body mass to determine if Surgery or Drug had any effects on weight. This analysis revealed a significant main effect of Surgery ($F(1,75)= 13.46$, $p<.001$; Table 3) but no effect of Drug ($F(1,75)= 0.03$, $p>.50$) or Surgery × Drug interaction ($F(1,75)= 0.14$, $p>.50$) on body mass. GDX subjects weighed less than Sham subjects. All together, these data suggest that normal T tone inhibits behavioural flexibility on a strategy set-shifting task primarily by promoting persistence. Furthermore, this data is unlikely to be explained by differences in memory for the initial visual-cue discrimination or differences in motivation or activity levels.

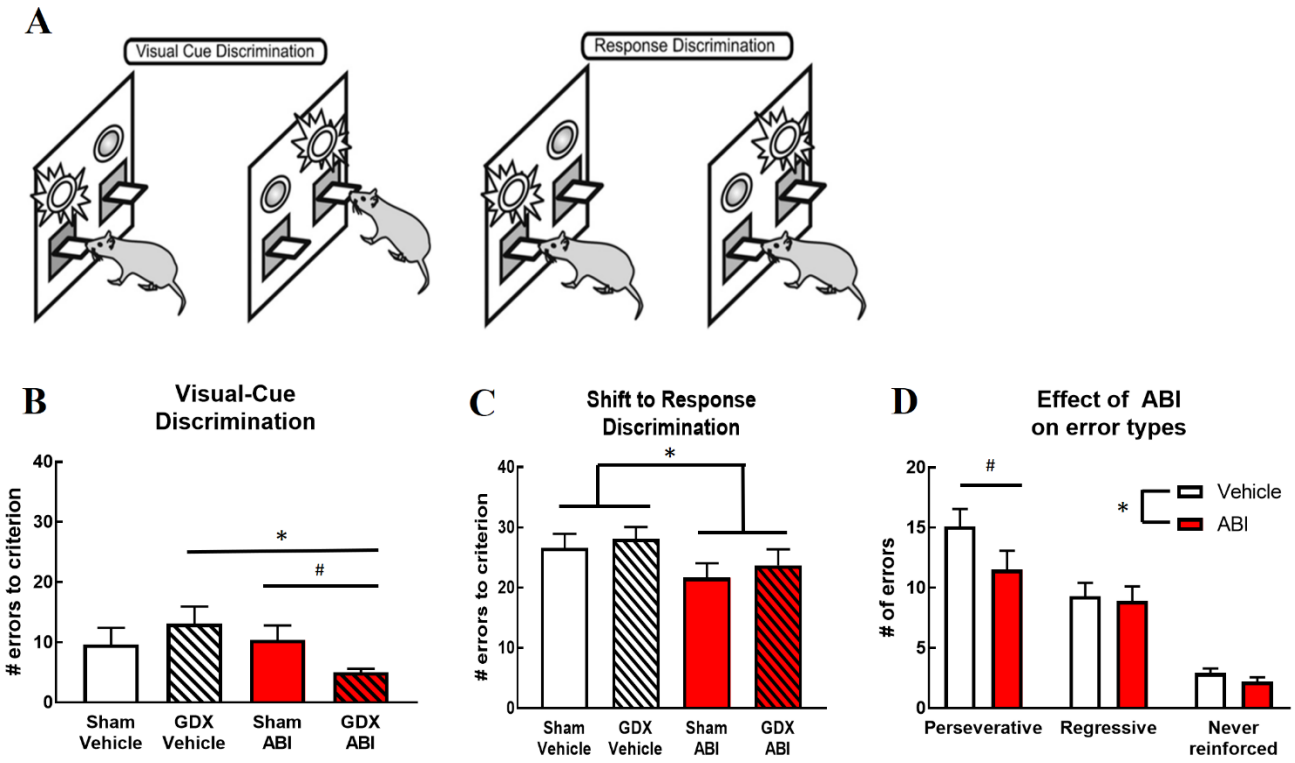

Fig. 5. Strategy Set-shifting. (A) Strategy set-shifting task in an operant chamber. First rats a trained on a visual-cue discrimination (left panel) which requires selection of the lever with the light above it to receive reward. Next rats are shifted to a response discrimination (right panel) which requires selection of one of the two levers (opposite of side bias; e.g. left lever) irrespective of light position to receive reward. Adapted from Floresco et al., 2008. (B) Total number of errors to criterion for the initial visual-cue discrimination. (C) Total number of errors to criterion for the shift to response discrimination. (D) Analysis of the types of errors made during the set-shift. Here data were collapsed across surgery groups to determine the effect of ABI on the error types. Data are expressed as means + S.E.M. $*p < .05$. $\#p < .10$.

Table 1. Selected behavioral parameters from strategy set-shifting. All data are means (SEM).

Behavioral Task	Sham-Veh	GDX-Veh	Sham-ABI	GDX-ABI
Study 1				
Strategy Set-Shifting				
Trials to criterion (visual-cue)	42.74 (6.84)	53.90 (8.41) a	51.45 (7.52) a	31.50 (0.75) b
Trials to criterion (Shift)	75.32 (5.85)	82.25 (5.32)	65.20 (5.57)	73.65 (6.61)
Cue-reminder correct (%)	82.37 (3.09) b	88.90 (1.25) a	85.74 (2.30)	81.74 (2.24) b
Omissions	0.211 (0.123)	0.450 (0.170)	0.250 (0.204)	0.100 (0.069)
Response latency (s)	0.54 (0.037) b	0.78 (0.085) a	0.53 (0.048) b	0.82 (0.096) a
Locomotor activity (beam breaks/min)	34.26 (4.19)	32.35 (2.25)	41.96 (3.91)	33.94 (2.95)

p <.05 a vs. b

Abiraterone acetate decreases perseveration during spatial reversal learning

Day 1: Response discrimination

Here we wanted to determine whether GDX or inhibition of androgen synthesis would affect response discrimination learning on a spatial reversal learning task. One animal from the GDX+ABI group was removed from behavioural analysis because the operant chamber malfunctioned. Analysis of these data revealed no main effect of Surgery ($F(1,59)= 0.02$, $p>.50$) or Drug ($F(1,59)= 0.00$, $p>.50$) and no Surgery × Drug interaction ($F(1,59)= 0.86$, $p=.36$; Table 2) on the number of trials to criterion. Furthermore, there was no main effect of Surgery ($F(1,59)= 0.07$, $p>.50$) or Drug ($F(1,59)= 0.46$, $p >.50$) and no Surgery × Drug interaction ($F(1,59)= 0.18$, $p>.50$; Fig. 6B) on the number of errors to criterion. Thus, all groups needed a similar amount of trials and made comparable amounts of total errors before reaching criterion on the initial response discrimination.

Day 2: Response reversal

Here we wanted to determine whether GDX or inhibition of androgen synthesis would affect behavioural flexibility through performance on a response reversal. First, we wanted to determine whether any effect would be driven by the ability to remember the initial response discrimination. Analysis from these data revealed no main effect of Surgery ($F(1,59)= 1.37$, $p=.25$) or Drug ($F(1,59)= 1.70$, $p=.20$) and no Surgery × Drug interaction ($F(1,59)= 2.07$, $p=.16$; Table 2) in the ability to remember the initial response discrimination. Thus, all groups were comparable in the ability to remember the initial response discrimination.

Analysis of trials to criterion for the response reversal revealed no main effects of Surgery ($F(1,59)= 1.07$, $p=.31$) or Drug ($F(1,59)= 0.13$, $p>.50$) and no Surgery × Drug interaction ($F(1,59)= 0.80$, $p=.38$; Table 2). Additionally, we wanted to investigate whether Surgery or Drug affected the types of errors being made during the response reversal, so we employed a between-within ANOVA design with Surgery and Drug as the between-subject factors and Error Type as the within-subject factor. This analysis revealed no effects of Surgery ($F(1,59)= 2.70$, $p=.11$) or Drug ($F(1,59)= 1.31$, $p=.16$) for the between-subject factors. However, there was a significant effect of Error Type ($F(1,59)= 72.87$, $p<.001$) and a significant Drug × Error Type interaction ($F(1,59)= 5.54$, $p<.05$). Thus, we analyzed the effect of Drug for each error type separately and found a significant effect of Drug on perseverative errors ($F(1,63)= 9.61$, $p<.01$) but no effect of Drug on regressive errors ($F(1,63)= 2.33$, $p=.13$; Fig. 6D). ABI subjects made fewer perseverative errors compared to Vehicle subjects and there was no difference in the amount of regressive errors between these groups.

To investigate whether these results were driven by motivation or activity levels we also analyzed response latencies, omissions and locomotor activity. Analysis from these data revealed no main effect of Surgery ($F(1,59)= 2.35, 0.42, 1.15$, NS) or Drug ($F(1,59)= 1.65, 2.11, 0.01$, NS; Table 2) on response latencies, locomotion, and omissions respectively.

Furthermore, we wanted to analyze differences in body mass to determine if Surgery or Drug had any effects on weight. This analysis revealed a significant main effect of Surgery ($F(1,59)= 39.41$, $p<.001$; Table 3) but no main effect of Drug ($F(1,59)= 0.04$, $p>.50$) or Surgery × Drug interaction ($F(1,59)= 0.00$, $p>.50$) on body mass. Thus, GDX subjects weighed less than Sham subjects.

Taken together, these data suggest that normal T tone works to promote persistence and has no effect on the ability to maintain a new strategy in the spatial reversal learning experiment. Furthermore, these data are unlikely to be explained by differences in memory for the initial response discrimination or differences in motivation or activity levels.

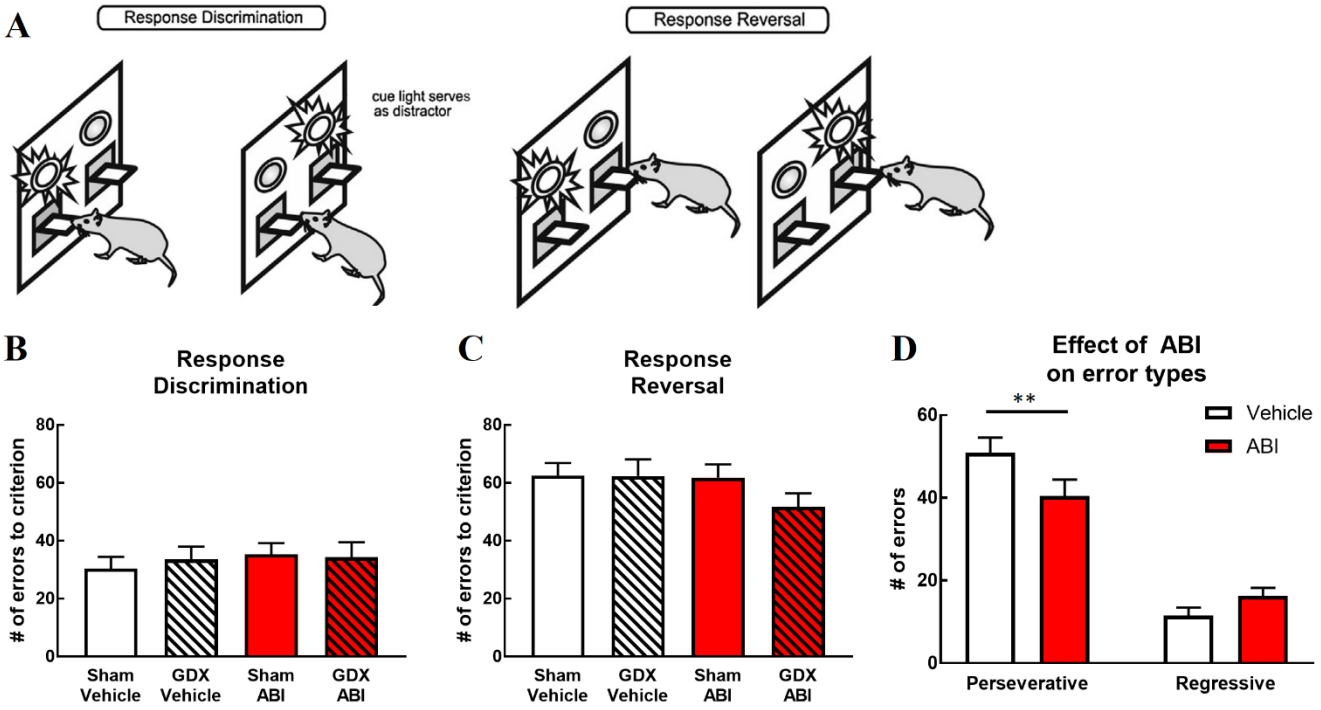

Fig. 6. Spatial Reversal Learning. (A) Reversal learning task in an operant chamber. Rats are trained on the initial response discrimination, which requires rats select the lever opposite of their side bias, irrespective of light position, to receive reward (left panel). Next, rats are shifted to another response discrimination, which requires selection of the opposite lever that was learned during the first response discrimination, irrespective of light position, to receive reward (right panel). Adapted from Butts, Floresco & Phillips, 2013. (B) Total number of errors to criterion for the initial response discrimination. (C) Total number of errors to criterion for the response reversal discrimination. (D) Analysis of the types of errors made during the response reversal. Here data were collapsed across surgery groups to determine the effect of ABI on the error types. Data are expressed as means + S.E.M.* $p < .05$, ** $p < .01$.

Table 2. Selected behavioral parameters from spatial reversal learning. All data are means (SEM).

Behavioral Task	Sham-Veh	GDX-Veh	Sham-ABI	GDX-ABI
Study 2				
Spatial Reversal Learning				
Trials to criterion (Response Discrimination)	76.56 (6.08)	83.56 (7.46)	82.50 (5.19)	77.33 (7.35)
Trials to criterion (Response Reversal)	106.81 (6.03)	105.88 (7.83)	110.25 (5.01)	97.80 (6.72)
Response-reminder correct (%)	77.19 (2.14)	76.56 (2.69)	76.88 (2.58)	83.00 (1.81)
Omissions	0.125 (0.125)	0.563 (0.273)	0.313 (0.254)	0.333 (0.159)
Response latency (s)	0.62 (0.043)	0.65 (0.060)	0.49 (0.030)	0.63 (0.080)
Locomotor activity (beam breaks/min)	32.87 (2.99)	35.08 (2.61)	33.06 (2.40)	27.59 (1.87)

p <.05 a vs. b

Table 3. Body mass from Study 1 & 2. All data are means (SEM).

	Sham-Veh	GDX-Veh	Sham-ABI	GDX-ABI
Stategy Set-Shifting (Study 1)				
Body mass (g)	381 (6) a	366 (5) b	380 (7) a	365 (5) b
Spatial Reversal Learning (Study 2)				
Body mass (g)	387 (5) a	359 (4) b	385 (4) a	358 (5) b

$p < .001$ a vs. b

Effect of gonadectomy and abiraterone acetate on serum and brain steroid levels

Testosterone

Serum

Here we wanted to validate that GDX and ABI effectively reduced T levels in serum of all subjects, including those undergoing transcardial perfusion. Analysis of the data in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,75)= 14.55$, $p<.001$) and Drug ($F(1,75)= 14.55$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,75)= 14.55$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the Vehicle-treated subjects ($F(1,75)= 28.71$, $p<.001$) and a significant effect of Drug in the Sham subjects ($F(1,75)= 28.71$, $p<.001$; Fig. 7A).

Analysis of the data in the spatial reversal learning experiment revealed a significant main effect of Surgery ($F(1,57)= 21.17$, $p<.001$) and Drug ($F(1,57)= 21.17$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,57)= 21.17$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the Vehicle-treated subjects ($F(1,57)= 43.17$, $p<.001$) and a significant effect of Drug in the Sham subjects ($F(1,57)= 43.17$, $p<.001$; Fig 7B). Thus, in both experiments, serum T was detectable in all Sham+Vehicle subjects but non-detectable in all subjects in the other groups.

mPFC

Analysis of mPFC T levels in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,36)= 15.60$, $p<.001$) and Drug ($F(1,36)= 15.60$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,36)= 18.14$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the Vehicle-treated subjects ($F(1,36)= 33.77$, $p<.001$) and a significant effect of Drug in the Sham subjects ($F(1,36)= 33.77$, $p<.001$; Fig. 7A).

Analysis of mPFC T levels in the spatial reversal learning experiment revealed a significant main effect of Surgery ($F(1,27)= 8.83$, $p<.01$) and Drug ($F(1,27)= 8.83$, $p<.01$) and a significant Surgery × Drug interaction ($F(1,27)= 8.83$, $p<.01$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the Vehicle-treated subjects ($F(1,27)= 18.24$, $p<.001$) and a significant effect of Drug in the Sham subjects ($F(1,27)= 18.24$, $p<.001$; Fig. 7B). Thus, in both experiments, mPFC T was detectable in all Sham+Vehicle subjects but non-detectable in nearly all subjects in the other groups.

DMS

Analysis of DMS T levels in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,36)= 22.16$, $p<.001$) and Drug ($F(1,36)= 20.49$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,36)= 20.49$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the Vehicle-treated subjects ($F(1,36)= 42.74$, $p<.001$) and a significant effect of Drug in the Sham subjects ($F(1,36)= 41.09$, $p<.001$; Fig. 7A).

Analysis of DMS T levels in the spatial reversal learning experiment revealed a significant main effect of Surgery ($F(1,27)= 10.84$, $p<.01$) and Drug ($F(1,27)= 10.84$, $p<.01$) and a significant Surgery × Drug interaction ($F(1,27)= 10.84$, $p<.01$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the Vehicle-treated subjects ($F(1,27)= 22.46$, $p<.001$) and a significant effect of Drug in the Sham-treated subjects ($F(1,27)= 22.46$, $p<.001$; Fig. 7B). Thus, in both experiments, DMS T was detectable in all Sham+Vehicle subjects but non-detectable in nearly all subjects in the other groups.

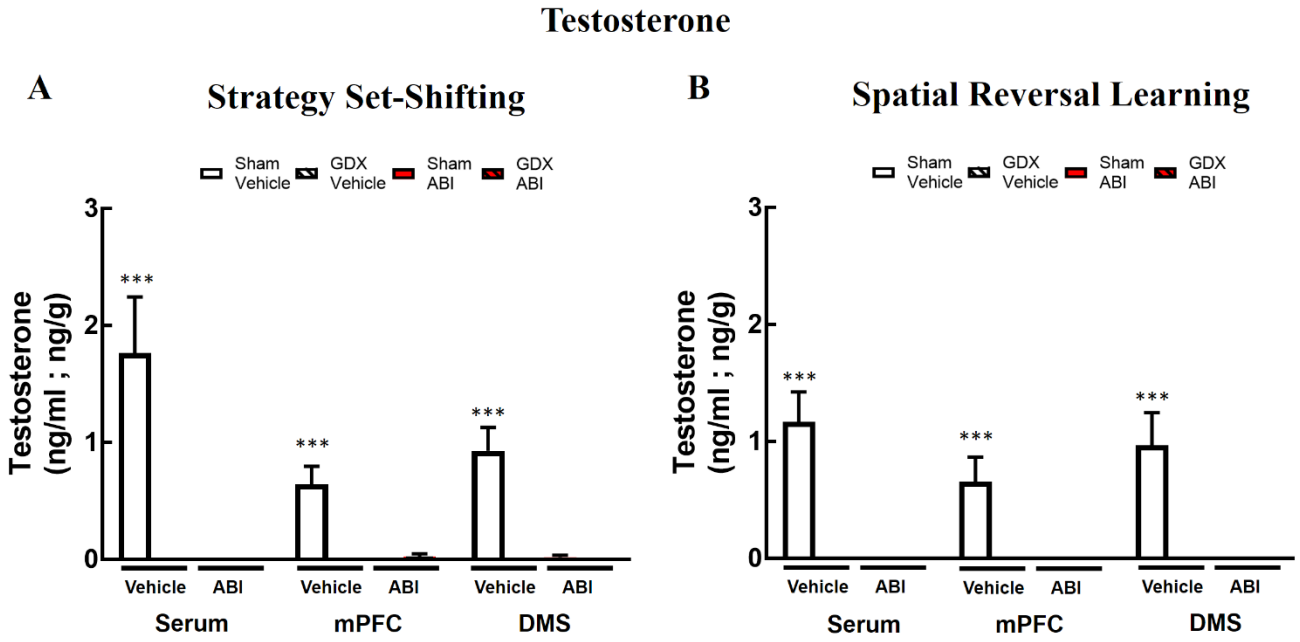

Fig. 7. Testosterone levels in Strategy Set-shifting and Spatial Reversal Learning experiments. (A) Testosterone levels (ng/mL for serum; ng/g for brain tissue) in serum, mPFC and DMS for the strategy set-shifting task. (B) Testosterone levels (ng/mL for serum; ng/g for brain tissue) in serum, mPFC and DMS for the spatial reversal learning task. Data are expressed as means + S.E.M. *** $p < 0.001$.

Corticosterone

Serum

Here we wanted to investigate whether our behavioural effects could be explained by differences in baseline CORT resulting from our GDX or ABI treatments. T can act as a brake on CORT production, and our manipulations may lead a disinhibition of CORT in the blood and in various brain regions (Handa et al 1994, Viau 2002). Analysis of serum CORT in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,75)= 4.13$, $p<.05$) but no main effect of Drug ($F(1,75)= 0.10$, $p>.50$) and no Surgery × Drug interaction ($F(1,75)= 0.00$, $p>.50$; Fig. 8A). Thus, in the strategy set-shifting experiment, GDX subjects had higher levels of serum CORT compared to Sham subjects.

Analysis of serum CORT in the spatial reversal learning experiment revealed a significant main effect of Surgery ($F(1,57)= 7.21$, $p<.01$) and Drug ($F(1,57)= 3.96$, $p=.05$) but no Surgery × Drug interaction ($F(1,57)= 0.10$, $p>.50$; Fig. 8B). Thus, in the spatial reversal learning experiment, GDX subjects had higher levels of serum CORT compared to Sham subjects, and ABI subjects had lower levels of serum CORT compared to Vehicle subjects.

mPFC

Analysis of mPFC CORT levels in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,36)= 7.82$, $p<.01$) but no effect of Drug ($F(1,36)= 0.11$, $p>.50$) and no Surgery × Drug interaction ($F(1,36)= 0.04$, $p>.50$; Fig. 8A). Thus, in the strategy set-shifting experiment, GDX animals had lower levels of mPFC CORT compared to Sham animals.

Analysis of mPFC CORT in the spatial reversal learning experiment revealed no main effect of Surgery ($F(1,27)= 0.49$, $p=.49$) or Drug ($F(1,27)= 0.42$, $p>.50$) and no Surgery × Drug interaction ($F(1,27)= 1.78$, $p=.19$; Fig. 8B). Thus, contrary to the strategy set-shifting experiment, subjects in the spatial reversal learning experiment had no significant differences in mPFC CORT levels.

DMS

Analysis of DMS CORT levels in the strategy set-shifting experiment revealed a significant effect of Surgery ($F(1,36)= 6.75$, $p<.05$) but no effect of Drug ($F(1,36)= 0.02$, $p>.50$) and no Surgery × Drug interaction ($F(1,36)= 0.09$, $p>.50$; Fig. 8A). Thus, in the strategy set-shifting experiment, GDX subjects had lower levels of DMS CORT compared to Sham subjects.

Analysis of DMS CORT levels in the spatial reversal learning experiment revealed no effect of Surgery ($F(1,27)= 1.93$, $p=.18$) or Drug ($F(1,27)= 0.37$, $p>.50$) and no Surgery × Drug interaction ($F(1,27)= 1.62$, $p=.21$; Fig. 8B). Thus, contrary to the strategy set-shifting experiment, subjects in the spatial reversal learning experiment had no significant differences in DMS CORT levels.

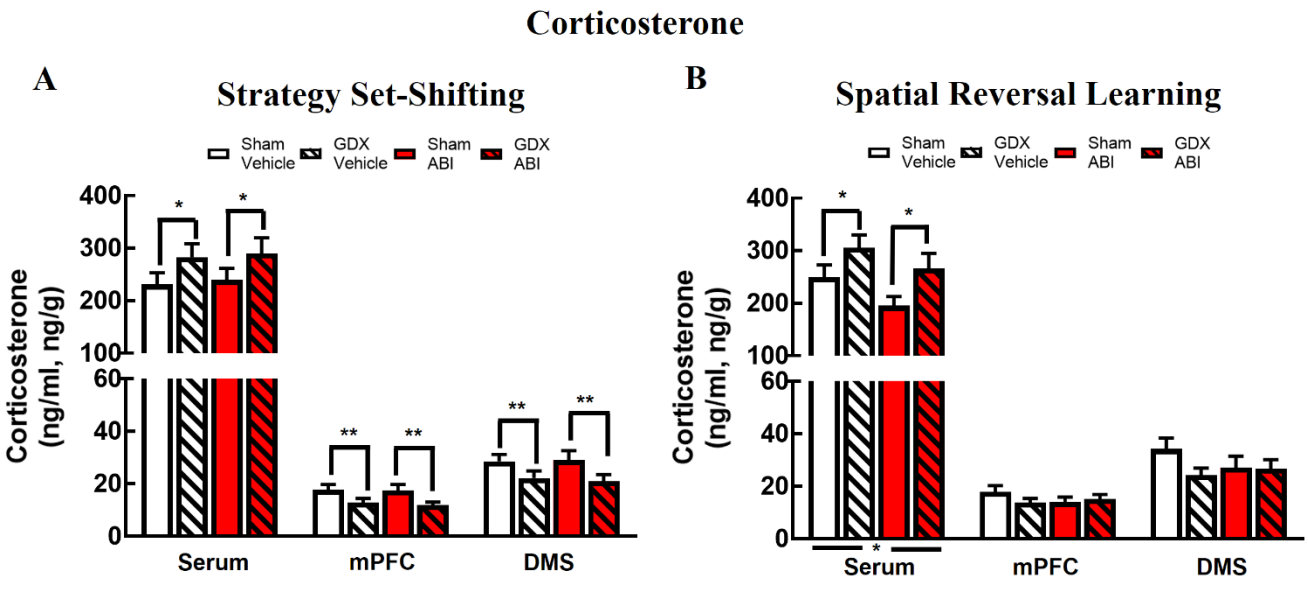

Fig. 8. Corticosterone levels in Strategy Set-shifting and Spatial Reversal Learning experiments. (A) Corticosterone levels (ng/mL for serum; ng/g for brain tissue) in serum, mPFC and DMS for the strategy set-shifting task. (B) Corticosterone levels (ng/mL for serum; ng/g for brain tissue) in serum, mPFC and DMS for the spatial reversal learning task. Data are expressed as means + S.E.M. * $p < 0.05$, ** $p < 0.01$.

Progesterone

Serum

Here we wanted to investigate whether our behavioural effects could be explained by differences in progesterone resulting from our GDX or ABI treatments. Progesterone is a precursor to T, and inhibiting androgen synthesis can lead to an increase of progesterone in serum and in the brain, especially for Sham animals. Analysis of serum progesterone in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,75)= 36.07$, $p<.001$) and Drug ($F(1,75)= 36.70$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,75)= 35.66$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in ABI-treated subjects ($F(1,75)= 72.67$, $p<.001$) and a significant effect of Drug in Sham subjects ($F(1,75)= 71.44$, $p<.001$; Fig. 9A).

Analysis of serum progesterone in the spatial reversal learning experiment revealed a significant main effect of Surgery ($F(1,57)= 23.48$, $p<.001$) and Drug ($F(1,57)= 23.67$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,57)= 27.12$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in ABI-treated subjects ($F(1,57)= 49.64$, $p<.001$) and a significant effect of Drug in Sham subjects ($F(1,57)= 51.66$, $p<.001$; Fig. 9B). Thus, in both experiments, serum progesterone was higher in Sham+ABI subjects compared to all other groups.

mPFC

Similar to our analysis in the serum, analysis of mPFC progesterone levels in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,36)= 17.72$, $p<.001$) and Drug ($F(1,36)= 17.24$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,36)= 17.17$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in ABI-treated subjects ($F(1,36)= 34.89$, $p<.001$) and a significant effect of Drug in Sham subjects ($F(1,36)= 34.41$, $p<.001$; Fig. 9A).

Analysis of mPFC progesterone levels in the spatial reversal learning experiment revealed a significant main effect of Surgery ($F(1,27)= 10.54$, $p<.01$) and Drug ($F(1,27)= 10.89$, $p<.01$) and a significant Surgery × Drug interaction ($F(1,27)= 9.89$, $p<.01$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the ABI-treated subjects ($F(1,27)= 19.74$, $p<.001$) and a significant effect of Drug in the Sham subjects ($F(1,27)= 21.50$, $p<.001$; Fig. 9B). Thus, in both experiments, mPFC progesterone was higher in Sham+ABI subjects compared to all other groups.

DMS

Analysis of DMS progesterone in the strategy set-shifting experiment revealed a significant main effect of Surgery ($F(1,36)= 12.52$, $p<.001$) and Drug ($F(1,36)= 12.20$, $p<.001$) and a significant Surgery × Drug interaction ($F(1,36)= 12.41$, $p<.001$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the ABI-treated subjects ($F(1,36)= 24.93$, $p<.001$) and a significant effect of Drug in the Sham-treated subjects ($F(1,36)= 24.61$, $p<.001$; Fig. 9A).

Analysis of DMS progesterone in the spatial reversal learning experiment revealed a significant main effect of Surgery ($F(1,27)= 11.07$, $p<.01$) and Drug ($F(1,27)= 11.00$, $p<.01$) and a significant Surgery × Drug interaction ($F(1,27)= 10.39$, $p<.01$). Subsequent analysis of the simple main effects found that there was a significant effect of Surgery in the ABI-treated subjects ($F(1,27)= 20.75$, $p<.001$) and a significant effect of Drug in the Sham-treated subjects ($F(1,27)= 22.16$, $p<.001$; Fig. 9B). Thus, in both experiments, DMS progesterone was higher in Sham+ABI subjects compared to all other groups.

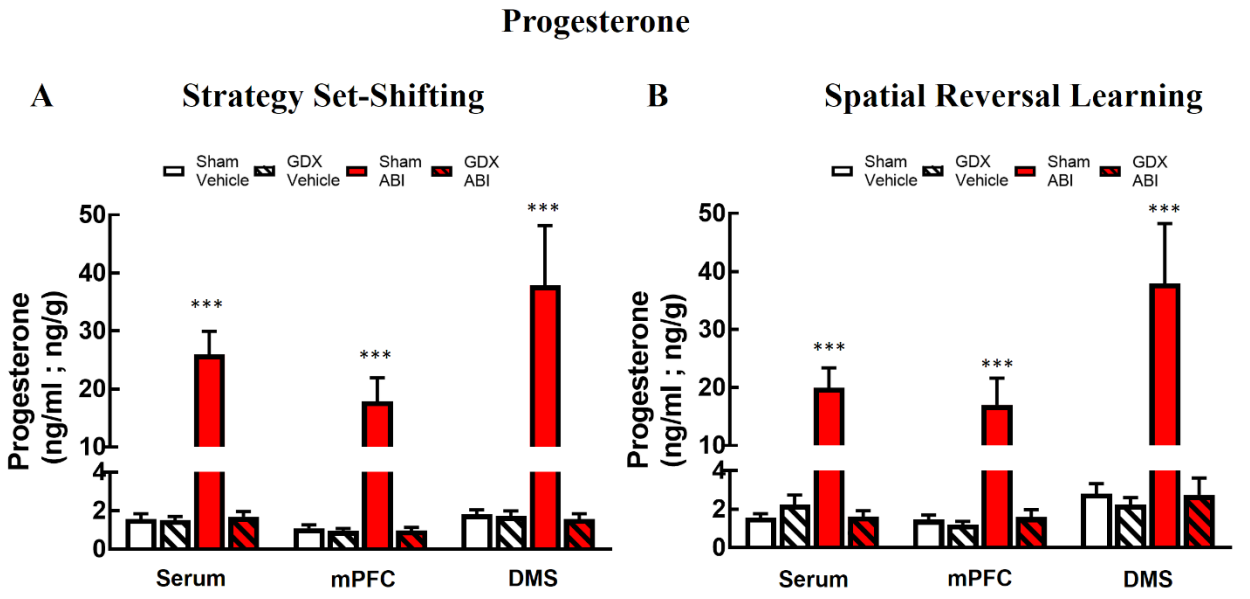

Fig. 9. Progesterone levels in Strategy Set-shifting and Spatial Reversal Learning experiments. (A) Progesterone levels (ng/mL for serum; ng/g for brain tissue) in serum, mPFC and DMS for the strategy set-shifting task. (B) Progesterone levels (ng/mL for serum; ng/g for brain tissue) in serum, mPFC and DMS for the spatial reversal learning task. Data are expressed as means + S.E.M. * $p < 0.05$, ** $p < 0.01$.

Estradiol and DHEA

E2 and DHEA were not detectable in the serum, mPFC or DMS of any animals.

DISCUSSION

The present studies provide evidence that GDX and ABI differentially affect behavioural flexibility. In the strategy set-shifting experiment, only ABI and not GDX improved behavioural flexibility by reducing the number of errors to criterion during the shift, suggesting GDX+Vehicle subjects maintain or increase local synthesis of androgens in the brain to maintain baseline behavioural flexibility. The effect of ABI in this experiment was driven by a modest reduction in perseveration for the initial visual-cue rule, indicating that ABI subjects disengage more readily from a previously correct, but now incorrect, strategy. In the spatial reversal learning experiment, neither GDX nor ABI affected behavioural flexibility, as indicated by the number of errors to criterion during the reversal. However, ABI did decrease perseverative errors during the response reversal without affecting regressive errors, indicating that ABI subjects disengage quicker from the previous rule but are no different in their ability to maintain a new rule. Additionally, T levels were effectively reduced by GDX and ABI in the serum, mPFC and DMS, suggesting that the behavioural effect of ABI might be driven by T synthesis in other brain regions involved in behavioural flexibility.

Abiraterone acetate improves strategy set-shifting performance

GDX and ABI differentially affected performance during the set-shift, suggesting that removing testicular androgens is different than inhibiting androgen synthesis both peripherally and centrally. Previous research shows that giving androgens to intact rats impairs set-shifting (Thompson & Wright 1979, Wallin & Wood 2015), whereas inhibiting androgen action (with cyproterone acetate) facilitates set-shifting (Thompson & Wright 1979). To our knowledge, there are no previous experiments that have investigated the effect of GDX on set-shifting performance

in male rats. Here we found that reducing androgen signaling with ABI, but not with GDX, facilitated set-shifting performance primarily through a reduction in perseveration. However, GDX+Vehicle subjects, which lack circulating T, perform similarly to Sham+Vehicle subjects during the set-shift. Indeed, GDX+Vehicle subjects had no measurable T in the serum, and traditionally it would be expected they would have no T in the brain. One possible explanation for the current data could be local synthesis of T in certain brain regions when systemic T levels are low (Fokidis et al 2015, Soma et al 2015). It could be that GDX+Vehicle subjects made a comparable number of errors during the set-shift as Sham+Vehicle subjects because brain regions involved in behavioural flexibility are able to locally synthesize or regulate their own androgens, even in the absence of gonadal production. Indeed, a variety of brain regions have the molecular machinery to synthesize or regulate steroids locally (Compagnone & Mellon 2000, Hojo et al 2004, Mellon & Deschepper 1993, Munetomo et al 2015). Brain T synthesis should only be inhibited by ABI treatment and could be conserved in GDX+Vehicle subjects within certain brain regions.

Interestingly, neither GDX nor ABI subjects had detectable T in the mPFC or the DMS, two brain regions critical to strategy set-shifting performance (Birrell & Brown 2000, Floresco et al 2008, Floresco et al 2006b, Floresco et al 2009, Ragozzino 2007, Ragozzino et al 1999a). This was surprising considering that at least in the mPFC, T is measurable in ~40% of long-term GDX male rats (Korol et al 2017). One possible explanation for this discrepancy might be that subjects in the current study were food restricted, which can decrease T levels in serum and mPFC (Govic et al 2008, Korol et al 2017, Levay et al 2010). Although we only measured steroids in two brain regions, there are other neural nodes important in regulating strategy set-shifting, and GDX+Vehicle subjects may have T present in regions like the nucleus accumbens (NAc) and ventral tegmental area (VTA).

The NAc is a brain region that mediates different aspects of strategy set-shifting and receives projections from the mPFC (Floresco 2015, Floresco et al 2006a). Interestingly, in one strategy set-shifting experiment that utilized adult male rats, inactivation of the NAc shell prior to the initial discrimination learning affected the ability to shift strategies, by facilitating the set-shift with a reduction in perseveration (Floresco et al 2006a). These findings are strikingly similar to the current data, and researchers have suggested that the NAc shell suppresses behaviour directed towards less profitable outcomes (Floresco 2015) and may encode information about irrelevant stimuli (Floresco et al 2006a). For example, during initial learning of the visual-cue, animals must not only learn that the visual-cue is predictive of reward, but must also learn that the absence of the visual-cue is predictive of reward omission. These parallel learning processes, one learning about relevant cues and one learning about irrelevant cues, are disrupted when the NAc shell is inactivated. Here we did not measure T in the NAc; however, data in our lab suggests that local T synthesis is high in this region (Korol et al 2017). Furthermore, steroidogenic enzymes are present in the NAc (Do Rego et al 2009, Korol et al 2017) and androgen receptors are higher in the shell than in the core sub-regions (Low et al 2017), which suggests a possible mechanism of androgens' effect on strategy set-shifting. Given previous findings, it could be that androgens normally work in the NAc shell to suppress behaviour directed towards less profitable outcomes, primarily through facilitating learning about irrelevant stimuli. If this is true, neural T may not only lead to increased persistence for the initial rule when strategies change, but may also prolong approach towards previously irrelevant strategies (e.g. "My key is not unlocking the door, maybe I should climb through the window…" "…No I should not, the window is always locked! I will try this key a few more times"). Thus, it is possible that when androgens in the NAc are inhibited, animals might be more inclined to shift quicker because they never really learned during the initial

discrimination that no light is predictive of reward omission (ie. learned irrelevance). ABI subjects would have a higher probability than Vehicle subjects to approach previously irrelevant cues (ie. sample options associated with no lights).

Another alternative that has been proposed is the synaptocrine hypothesis of steroid signalling in the brain (Saldanha et al 2011). There is evidence that suggests sex steroids like E2 can act like neurotransmitters and it is proposed that E2 is rapidly synthesized in presynaptic boutons, and after release and action, it is metabolized rapidly in the synapse cleft (Saldanha et al 2011). Indeed, membrane-bound estrogen receptors can have rapid effects on cell function (Brailoiu et al 2007). Furthermore, it has been proposed that androgens have membrane-bound receptors that can have rapid effects on cellular function (Heinlein & Chang 2002). In the current study, we were unable to detect E2 in any of the subjects; however, with the current methods available it is difficult to detect E2 in the male rat brain without larger amounts of tissue. It is possible that T or E2 could be having synaptocrine actions within circuits important for behavioural flexibility in GDX+Vehicle subjects, but given the current methods it is difficult to test this hypothesis. Indeed, future studies would have to clarify if synaptocrine signalling might be inhibited in the ABI treated animals during strategy set-shifting.

Interestingly, in Experiment 1 we found that GDX+ABI subjects were quicker at learning the visual-cue and experienced fewer trials compared to GDX+Vehicle subjects. It could be argued that weaker encoding of the initial visual-cue rule is driving the effect of ABI during the shift. We find this explanation unlikely given that Sham+ABI subjects were similar to the control Sham+Vehicle subjects during visual-cue learning. Furthermore, Sham+ABI subjects performed the best of all groups during the shift. Additionally, if GDX+ABI subjects were better at shifting strategies due to weaker encoding for the initial rule, then you would expect memory of the visual-

cue in the reminder trials to be lower than control. However, this is not the case because both ABI groups performed similarly to the control Sham+Vehicle subjects during the reminder trials.

T can inhibit CORT secretion (Handa et al 1994, Viau 2002) and reducing T levels with GDX or ABI could lead to a disinhibition of CORT release in the blood and in the brain. CORT is primarily known as a stress hormone, and stress can affect set-shifting performance (Butts et al 2013). Thus, we wanted to investigate whether disinhibition of CORT could be driving the effects we observed. In the strategy set-shifting experiment, we found that GDX increased CORT in serum but ABI had no effect. Interestingly, CORT levels are much higher in the serum than in the brain, showing that serum steroid levels do not always reflect brain steroid levels. Furthermore, GDX reduced CORT levels in the mPFC and DMS, which was opposite to what GDX did in the serum. ABI had no effect on CORT levels. Given this pattern of results, we find it unlikely that changes in CORT in the blood or the brain are driving our effects because you would expect only an effect of ABI if it were to match the pattern observed during the set-shift.

CYP17A1 is an enzyme that catalyzes the reactions involved in converting progestins into androgens (Akhtar et al 2011). The gonads contain high levels of CYP17A1 in male rats, which can lead to an accumulation of upstream progestins when the androgen pathway is blocked by ABI, especially in the gonadally intact animals. Thus, we wanted to investigate if increased progesterone in the blood or brain could be driving the effects observed in the strategy set-shifting experiment. As expected, progesterone levels were elevated in the serum, mPFC, and DMS of SHAM+ABI subjects compared to all other groups. More importantly, there was no elevation of progesterone in GDX+ABI subjects. Thus, we find it unlikely that progesterone could be driving the performance during the set-shift because you would expect both ABI groups to have similar levels of progesterone and this is not the case.

It is important to note that GDX increased response latencies during the set-shift but ABI had no effect. Increased response latencies are typically indicative of decreased motivation; however, we find it unlikely that changes in motivation are driving performance during the set-shift because all groups were similar in the number of omissions made. Similarly, GDX decreased body mass but ABI had no effect, suggesting again that performance during the set-shift are unlikely to be explained by differences in weight.

In many cases, GDX and ABI had different effects on a variety of measures on this task. Taken together, these data suggest that gonadally-produced androgens and neurally-produced androgens may have differential effects on neurophysiology, providing indirect evidence for neurosteroid functions. For example, ABI but not GDX affected errors to criterion during the set-shift. In contrast, GDX but not ABI affected response latencies. The exact mechanisms that give rise to the behavioural differences when systemic or local androgens are manipulated remain to be determined.

Abiraterone acetate decreases perseveration during spatial reversal learning

Previous research has shown that giving androgens to intact young male rats impairs reversal learning (Wallin & Wood 2015); however, research is lacking looking at the inhibition of androgens during reversal learning. One research group reported no effect of GDX in male rats during reversal learning when a match to sample task was reversed to a non-match to sample task (Kritzer et al 2007). Although this is consistent with the current study, it is important to note that interpretation of the Kritzer et al (2007) experiment is complicated by the working memory component required for match/non-match to sample tasks. It is suggested that reduced T levels facilitate extinction (Brownson et al 1994, Chambers et al 1993). Extinction is the most basic form of flexible behaviour because organisms must first extinguish from previously conditioned

responses before they can adjust behaviour to more profitable options. Consistent with previous reports (Kritzer et al 2007), we showed that neither GDX nor ABI had any effects on the total errors to criterion during the initial response discrimination or during the response reversal. However, ABI subjects did have decreased perseveration compared to Vehicle subjects during the response reversal, suggesting that inhibiting androgens resulted in quicker extinction or suppression of the initial rule, even though it did not result in improved performance. Furthermore, there was no effect of GDX on perseverative errors which suggests that androgens may be upregulated in brain regions involved in reversal learning to maintain baseline function. Collectively this suggests that normal T tone may work to promote persistence and prolong suppression for previously correct but now incorrect rules.

The reduction in perseveration in ABI subjects is unlikely due to decreased memory for the initial rule because all groups performed similarly during the reminder trials. Additionally, differences in motivation and activity levels are unlikely to explain the decrease in perseveration from ABI treatment because all groups had similar response latencies, omitted trials and locomotor activity.

For the reasons described above we wanted to see if the reduction in perseveration from ABI in the spatial reversal learning experiment could be explained by T, CORT, or progesterone. Neither GDX nor ABI subjects had measurable T in the serum, mPFC or DMS, which was surprising because we expected GDX+Vehicle subjects to have increased local T synthesis in these regions. Although the DMS is important in regulating reversal learning (Ragozzino 2007), the LOFC is another brain region that is critical in regulating reversal learning (Boulougouris et al 2007, Floresco et al 2009, Ghods-Sharifi et al 2008, Kim & Ragozzino 2005, Schoenbaum et al 2002). Steroids were not measured in the LOFC and GDX+Vehicle subjects may have T present

in this brain region. If this is the case, it might explain why we found no effect of GDX on perseverative errors during the response reversal.

There was an effect of both GDX and ABI on serum CORT levels, where GDX increased CORT in the serum and ABI reduced CORT in the serum. It could be argued that the effect of ABI in serum CORT is driving the effects of ABI on perseveration. However, we find this explanation unlikely because there was no difference between all groups in mPFC or DMS CORT levels. It is important to note that this does not rule out that ABI could be affecting CORT levels in other brain regions.

Finally, as expected, progesterone levels were elevated in the serum, mPFC, and DMS of SHAM+ABI subjects compared to all other groups. More importantly, there was no elevation of progesterone in GDX+ABI subjects. Thus, we find it unlikely that progesterone could be driving the effect on perseveration because you would expect both ABI groups to have similar levels of progesterone and this is not the case.

Similarities and differences between experiments

Although there was a consistent effect of ABI in both experiments with a reduction in perseveration, there are some differences between experiments that must be addressed. For example, during the set-shifting experiment, GDX+ABI subjects were quicker to learn the initial visual-cue discrimination but in the spatial reversal learning experiment all groups were similar in their ability to learn the initial response discrimination. Although this may seem conflicting, this provides support that ABI's effect during strategy set-shifting is the result of specific alterations in behavioural flexibility and not due to differences in response learning. It could be argued that ABI animals are better response learners which could explain why they made less errors during

the set-shift. However, we find this unlikely given that ABI animals in the spatial reversal learning experiment, experienced all the same manipulations as the strategy set-shifting animals, but were similar to the other groups in their ability to learn a response rule.

Another difference between experiments was differences in serum and brain CORT levels. In the strategy set-shifting experiment, GDX increased serum CORT compared to Sham subjects. However, in the spatial reversal learning experiment GDX and ABI had opposing effects, where GDX increased but ABI decreased serum CORT. This is surprising because you would expect ABI to reduce androgens, leading to increased CORT secretion (Handa et al 1994, Viau 2002). However, considering that animals in both experiments were treated similarly, it is puzzling that we found this difference in spatial reversal learning animals. Regardless, animals in the spatial reversal learning experiment had no differences in mPFC or DMS CORT, which suggests the decrease in perseverative responding from ABI is not due to differences in neural CORT. However, it is important to note that neural CORT levels were different in the strategy set-shifting task, with GDX animals having lower CORT levels in the mPFC and DMS. This discrepancy between experiments suggests that local regulation of neural CORT may be different depending on the task being examined. For example, the mPFC is critical for set-shifting but not for reversal learning (Floresco et al 2008). It could be that brain regions critical in a task, when activated, results in rapid changes in the local regulation of steroids within that brain region. If this is the case then you might expect the spatial reversal learning animals to have differences in neural CORT within the LOFC, a brain region important for reversal learning. However, we are only proposing this because there have been no studies that examine how neural activation during tasks of executive function might affect the local regulation of steroids.

It is important to note that there were more similarities than differences between studies, which makes the pattern of results more convincing. For example, in both studies the T, CORT and progesterone in the serum and brain of Sham+Vehicle subjects, fell within the normal physiological ranges found in other rat studies (Korol et al 2017, Taves et al 2011b). Furthermore, T and progesterone in the serum and brain showed similar patterns to their respective groups in both studies. Although we did not find neuroT synthesis in the GDX+Vehicle subjects as we expected, it is likely that no effect of GDX on behavioural flexibility is due to increased local androgen synthesis in other brain regions critical to regulating these behaviours. This does not rule out that ABI may be having off-target effects on neurophysiology. ABI can inhibit androgen receptor activity (Soifer et al 2012, Yin & Hu 2014); however, these effects are only observed when higher doses of the drug are administered. The dose of ABI used in the current study (40mg/kg) make these off-target effects highly unlikely.

Possible mechanisms of androgens effects on strategy set-shifting and spatial reversal learning

The DA system is comprised of two predominant circuits as it relates to reward related decision making and executive function. The mesocortical and mesolimbic circuits send projections from the VTA to cortical and limbic regions respectively, including the mPFC and NAc. The neuromodulation of DA in these regions are critical in regulating strategy set-shifting (Floresco et al 2006b, Haluk & Floresco 2009). Furthermore, the mPFC shows sex differences in the regulation of DA release to drug challenges (Locklear et al 2016), suggesting a role of sex steroids in regulating DA effects on strategy set-shifting. Indeed, androgens have been shown to affect mRNA expression for different proteins important for DA synthesis, breakdown and transport (Purves-Tyson et al 2014). The DA synthetic enzyme tyrosine hydroxylase (TH) is affected by GDX and may increase or decrease, depending on the brain region and GDX duration

(Kritzer 2000, Kritzer 2003, Kritzer et al 2007). For example, short-term (~4 d) GDX leads to a downregulation of TH in cortical areas that is reversed by E2 but not the non-aromatizable androgen DHT. In contrast, long-term (~28 d) GDX leads to an upregulation of TH in cortical areas that is reversed by DHT but not E2 (Kritzer 2000). T and E2 modulate NMDA and AMPA dependent DA release in the mPFC (Aubele & Kritzer 2011, Aubele & Kritzer 2012) and increase DA release in the NAc and striatum (de Souza Silva et al 2009, Hernandez et al 1994). Given the important role of DA in regulating strategy set-shifting (Floresco et al 2006b, Haluk & Floresco 2009) and the role of androgens in modulating the DA system, we find it likely that the effects in the strategy set-shifting experiment may be driven mainly by androgen modulation of cortical and subcortical DA. Indeed, different DA-ergic markers will need to be measured in these animals to clarify if androgens effect on the strategy set-shifting task is being driven by alterations in DA.

The serotonin (5-HT) system is comprised of neurons originating in the raphe nucleus which project throughout the cerebral cortex and limbic regions, including the OFC. Indeed, 5-HT signalling is important for regulating reversal learning (Clarke et al 2005) and androgens and estrogens have receptors in 5-HT neurons within the raphe nucleus (Sheng et al 2004). Furthermore, anabolic androgenic steroids increase 5-HT-ergic activities within the rat and mouse brain (Ambar & Chiavegatto 2009, de Souza Silva et al 2009, Kindlundh et al 2003). Given the important role of 5-HT in regulating reversal learning and the role of androgens in modulating the 5-HT system, we find it likely that the effects in the spatial reversal learning experiment may be driven mainly by androgen modulation of the 5-HT system. However, it is important to note that 5-HT-ergic markers will also have to be measured in these animals to determine if androgens are affecting this system.

Conclusions

Suppression of androgens with GDX or ABI had different effects on behavioural flexibility, suggesting that testicular androgens may have different effects on neurophysiology than neurally-produced androgens. In both experiments, ABI either improved behavioural flexibility by reducing the number of errors to criterion during the set-shift, or by decreasing perseveration during the response reversal, suggesting that T promotes persistence for a learned strategy or rule. The null effect of GDX on the errors to criterion during the set-shift or perseverative errors during the response reversal, suggests that neural steroid synthesis is occurring in the GDX+Vehicle subjects to help maintain baseline flexibility. Although there was no detectable T in the mPFC or DMS of GDX+Vehicle subjects, we find it likely that the behavioural effects are the result of local androgen synthesis in other brain regions important for behavioural flexibility. The current data provide important insights into neural steroid synthesis and into the effects of androgens on behavioural flexibility. Furthermore, these data provide important clinical insights into changes that might occur in executive function in prostate cancer patients receiving ABI.

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