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Altered Sensitivity to Cocaine in Adolescent Spontaneously Hypertensive Rats, A Rodent Model of Attention-Deficit/ Hyperactivity Disorder

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BY

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Altered Sensitivity to Cocaine in Adolescent Spontaneously Hypertensive Rats, A Rodent Model of Attention-Deficit/Hyperactivity Disorder

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders diagnosed in childhood. It is characterised by an inability to remain still, an inability to maintain focus, and impaired self-control (CDC, 2021). The DSM-V currently recognises three presentations based on the main symptoms of the individual, those being predominantly inattentive (ADHD-I), predominantly hyperactive-impulsive (ADHD-HI), and combined (ADHD-C; American Psychiatric Association & American Psychiatric Association, 2013). In 2007, it was estimated through systematic review and metaregression analysis that ADHD affects approximately 5% of individuals globally (Polanczyk et al., 2007). Concerningly, however, the prevalence of this disorder has been increasing. Data collected from 2013-2019 revealed approximately 1 in 11 adolescents aged 3-17 were affected by ADHD in the U.S. (Bitsko et al., 2022). With this increase in prevalence, a need to further evaluate the consequences of ADHD on the individual grows in importance. A systematic review of studies on academic performance and achievement test scores showed individuals with ADHD score consistently lower than their counterparts (Arnold et al., 2020). Additionally, a longitudinal study reported lower reading ability and cognitive skills in preschoolers identified as hyperactive that persisted 12 years after the initial evaluation (McGee et al., 1991), showing these cognitive impairments follow an individual from a very young age, through adolescence and often into adulthood. As ADHD can be symptomatic as young as six years old, this academic impairment can cause significant detriment to an individual's education. Indeed, individuals with ADHD have been shown to have completed an average of 2 years less education than their non-ADHD

counterparts by the age of 24 (Mannuzza et al., 1997). Importantly, children with increased hyperactivity scores demonstrated higher levels of delinquent behaviour and trouble in school later in adolescence (McGee et al., 2002), and a higher prevalence of comorbid psychiatric disorders including alcohol and substance use disorders (Anker et al., 2018). This demonstrates the negative consequences of ADHD stem beyond education and employment.

However, there has been conflicting literature regarding how ADHD may affect males and females differently in diagnostic rates, symptomology, and symptom progression. A twin study on diagnosis rates in 9-12-year-olds revealed males are more frequently diagnosed with ADHD overall, with the majority being ADHD-C, whereas females are more frequently diagnosed with ADHD-I (Mowlem et al., 2019). Likewise in symptomology, a similar twin study in 8-19-year-old individuals found more males to have impairments in inattention, hyperactivity/impulsivity, and overall ADHD symptomology than females (Arnett et al., 2015). This is further supported by a 2005 study that determined males had higher rates of inattentive and hyperactive/impulsive symptoms than females (F. Levy et al., 2005). Interestingly, however, a study on college-aged individuals determined that in individuals without ADHD, women reported lowered levels of inattention and hyperactivity than men, whereas in individuals with ADHD, women reported higher levels of inattention and hyperactivity than men (Fedele et al., 2012). This study also reported impairment scores for individuals with ADHD, with women endorsing a significantly greater impairment in home life, education, money, social life, and overall impairment than males. It is also the case that some studies have reported no difference in ADHD symptom severity among the sexes (Günther et al., 2015, Rasmussen & Levander, 2009). A potential reason is revealed in a study that evaluated both inattentive and hyperactivity/impulsivity symptoms' developmental trajectories for each sex independently,

which revealed sex differences in the development of both ADHD symptoms (Murray et al., 2019). Considered together, this disagreement in the present ADHD literature points towards a need for further investigation into the sex differences in ADHD over the progression of time.

To limit the symptomatology associated with ADHD and its cognitive detriments, ADHD is commonly treated using a combination of psychotherapy and psychostimulants. These medications have been demonstrated to improve, but not cure, the impairments of ADHD and boost classroom behaviour and performance (Advokat et al., 2011; Evans et al., 2001). Due to their efficacy and ease, medication-based treatment of ADHD has quickly become immensely popular. A study conducted from 2000-2005 revealed a rapid increase in the use of ADHD medications in adults and children, with a 9.5% annual growth rate of stimulant use for children (0-19 years old; Castle et al., 2007). Of the individuals sampled, approximately 50% were receiving methylphenidate. This drug, also known as Ritalin, is commonly used as the first-line medication to treat ADHD, likely due to its FDA safety approval in children as young as 6 years old. Methylphenidate is considered safer than other psychostimulants, with no increased cardiovascular-related events, though it has been shown to predict a higher body mass index and may affect metabolism long-term (Bowling et al., 2017; Habel et al., 2011). Using functional magnetic resonance imaging (fMRI) studies, methylphenidate has been demonstrated to increase the dopamine availability in the synapse significantly and is believed to block the dopamine transporter in the striatum of the brain (Volkow et al., 2005). Positron emission tomography (PET) imaging of the brain when administered methylphenidate demonstrates decreased dopamine receptor availability (Volkow et al., 2002). However, meta-analyses of sex differences among pharmacotherapy revealed that females with ADHD are less likely to be prescribed drug treatments for their symptoms (Kok et al., 2020). Additionally, this study revealed sex

differences in the effects of methylphenidate on children under 12. In children using methylphenidate, females had significantly better ability to focus attention than males, but had poorer academic competency improvement. This altered performance using a stimulant treatment points towards a sex difference in the neural underpinnings of ADHD.

As highlighted above, ADHD medications such as methylphenidate exert their therapeutic effects via direct targeting of brain dopamine pathways such as the mesocorticolimbic dopamine system. This system mediates the experience of reward. This pathway originates from cell bodies located in the ventral tegmental area (VTA) in the midbrain, which has been determined to receive inputs from a variety of sources - serotonin, oxytocin, glutamate, and GABA - and release not only dopamine but GABA and glutamate to various locations in the brain (Beier et al., 2015). Mesocorticolimbic dopaminergic neurons project from the VTA to terminate in brain areas involved in reward and motivation, including the nucleus accumbens and the prefrontal cortex (Settell et al., 2017). In the nucleus accumbens and prefrontal cortex, medium-spiny neurons exhibiting dopamine D1 and D2 receptors mediate the effects of psychostimulants (Scofield et al., 2016). Following the binding of dopamine extracellularly, these G-protein coupled receptors have been found to exert either primarily stimulating, in the case of D1 receptors, or primarily inhibitory, in the case of D2 receptors, responses (Scofield et al., 2016). When the dopaminergic pathway to the nucleus accumbens was destroyed, previously stable self-administration rates of cocaine significantly decreased, demonstrating dopaminergic signalling in the nucleus accumbens is necessary for addiction to this drug (Gerrits & Van Ree, 1996). In the nucleus accumbens, dopamine D1 receptors have been demonstrated to play a role in the experience of reward, as demonstrated by a D1 receptor antagonist decreasing a rat's motivation to work for food, water, and saccharin (Sanger, 1987).

Furthermore, the mesocorticolimbic dopaminergic neurons that terminate in the prefrontal cortex are believed to be responsible for motivation and reward learning signals, as evidenced by fMRI studies in humans receiving points rewards based on their performance in a force measure task (Hauser et al., 2017). Similarly, the administration of a dopamine D1 receptor antagonist into the medial prefrontal cortex decreases in the breakpoint, the number of responses performed to receive the final self-administration of cocaine, demonstrating a decrease in the reinforcing effects of cocaine (McGregor & Roberts, 1995). Together, following the administration of a drug of abuse, dopamine released from neurons projecting from the VTA binds to D1 receptors in the nucleus accumbens and the prefrontal cortex to cause the experience of pleasure and reinforcement.

Although there is no single cause underlying all instances of ADHD, alterations to the mesocorticolimbic dopamine system may be one common factor. The high heritability of ADHD suggests a genetic component to the disorder, with the DAT1 gene, a gene that codes for the dopamine transporter (DAT), having been implicated as a potential cause (Franke et al., 2010). The DAT is responsible for regulating dopamine transmission in the brain by promoting the reuptake of dopamine (Vaughan & Foster, 2013). This mechanism decreases the amount of dopamine available to signal by pulling excess neurotransmitters back into the presynaptic neuron from which it was released. In patients with ADHD, an fMRI study revealed increased DAT density relative to control, indicating a neural difference in individuals with ADHD (Dougherty et al., 1999). Increased transporters in ADHD could lead to less dopamine availability, and this could underlie symptomatology. Indeed, the spontaneously hypertensive rat (SHR), a rodent model of ADHD, exhibited significantly decreased release of dopamine in the prefrontal cortex and the caudate putamen following electrical stimulation, though this was not

observed in the nucleus accumbens (Russell et al., 1995). Aside from the genetic heritability of the overexpression of the DAT1 gene, prenatal exposure to cigarette smoke has been shown to potentially exacerbate the hyperactivity and impulsivity symptoms of a child with a homozygous for a DAT1 impairment, though this was only significant in males (Becker et al., 2008). This suggests a complicated cause of ADHD between genetics and environment that could play a role in the presentation of symptomatology.

It is not surprising that individuals with ADHD could choose to self-medicate with an illicit stimulant such as cocaine, given the similarities in mechanism of action between these drugs and licit stimulants prescribed for ADHD such as methylphenidate. A meta-analysis of stimulant use disorders in individuals with ADHD reveals an estimated prevalence of cocaine use in adults with ADHD as 26.0% and estimates approximately one in ten develop a cocaine use disorder in their lifetime (Oliva et al., 2021). A PET study performed in mice determined cocaine and methylphenidate have similar dose-dependent dopamine transporter occupancy, indicating the mechanism by which cocaine affects dopamine is quite similar to methylphenidate (Gatley et al., 1999). Furthermore, a comparison PET study of the effects of cocaine and methylphenidate revealed similarly rapid increases of dopamine in the brain, and that these drugs competed for the same binding sites (Volkow et al., 1995). However, in the case of cocaine, the clearance of this drug from the striatum was significantly faster than the clearance of methylphenidate, meaning the effects of cocaine were much shorter. This makes cocaine a much riskier option for individuals with ADHD who use this drug as a means for ameliorating their symptoms, as its effects are short-lived compared to methylphenidate.

With adolescence being a time during which many individuals choose to initiate drug use, substance use disorders are exceptionally concerning during this time. For those with ADHD,

this risk may be even greater. A longitudinal study of those with ADHD found they were significantly more likely to be diagnosed with a substance use disorder at this time, and were more likely to remain dependent on drugs or alcohol later into adulthood (S. Levy et al., 2014). This increase in substance use prevalence is worrying, as adolescence is a period that has immense consequences on the trajectory of one's life. These are dramatic hormone and brain developmental changes as the individual undergoes puberty, making this a critical organisation window (Vigil et al., 2016). Although the prevalence of illicit substance use in adolescents with ADHD is established, existing preclinical literature on ADHD and stimulant use has been focused on adult subjects; thus little is known about how cocaine may be differentially experienced by adolescents with ADHD. Additionally, sex differences have been demonstrated in the development and progression of psychostimulant addiction, with women having a higher prevalence of substance use disorders and suffering from relapse at higher rates (Kokane & Perrotti, 2020). Underlying this sex difference may be the differences in sex steroid hormones among males and females. In female rats, the fluctuations of estradiol and progesterone have been mapped onto variations in the self-administration of cocaine. Rats in the mid-estrus period, equivalent to the early luteal phase just following ovulation in the human menstrual cycle, showed heightened reinstatement of cocaine-primed drug-seeking following the extinction of self-administration (Kippin et al., 2005). Adolescence, in particular, is a period during which dramatic changes in sex steroid hormones have significant impacts on brain development, with MRI data demonstrating significant sex differences in cortical and subcortical brain volumes between male and female adolescents (Herting et al., 2014). While these dramatic changes in sex steroid hormones caused by puberty have been known to differentially impact the development

and progression of psychostimulant addiction in males vs. females, no one has examined whether there are sex differences in the effects of psychostimulants in adolescents with ADHD.

Rodent models have been developed to promote the understanding of disorders like ADHD, including the spontaneously hypertensive rat (SHR). Originally derived from Wistar-Kyoto rats, this strain was selectively bred for its hypertensive phenotype (Okamoto & Aoki, 1963), and has been validated in a variety of studies as a model of ADHD (Sagvolden, 2000). Adolescent male SHRs had a further distance travelled in a 20-minute open-field test than control strains (de la Peña et al., 2013), demonstrating significant hyperactive tendencies in this rat strain. Additionally, in a test of attention, adolescent male SHRs performed significantly less spontaneous alternations than the reference strain, indicating impaired working memory as is seen in ADHD (de la Peña et al., 2013). Furthermore, SHRs have been shown to have an altered dopaminergic system similar to humans with ADHD. When stimulated with microelectrodes, dopamine release in SHRs was significantly decreased versus a control strain, indicating decreased dopamine availability similar to humans with ADHD (Miller et al., 2012). This study further examined the dopamine uptake speeds in SHRs as a functional measure of the dopamine transporter's performance and found a significantly faster uptake in the ventral striatum and nucleus accumbens in SHRs (Miller et al., 2012). These behavioural and neural congruences support the spontaneously hypertensive rat as a rodent model of ADHD.

Current research in SHRs has predominantly focused on adult male rats, with few studies evaluating the adolescent period and susceptibility to drugs of abuse like cocaine. The present study sought to extend research in adolescent drug use in individuals with ADHD to elucidate potential sex differences in the behavioural and neural response to cocaine in SHRs. We hypothesised that an altered mesocorticolimbic dopamine reward pathway in individuals with

ADHD underlies the increased risk of developing substance use disorders in adolescence in this population. Given the psychoactive effects of cocaine are mediated (in part) by increases in dopamine signalling in the brain, we expected the altered dopamine pathway in SHRs to trigger enhanced neural and behavioural responses following injection of cocaine. Behaviourally, we expected that SHRs would exhibit an enhanced cocaine-induced locomotor response on the challenge day of a behavioural sensitisation protocol than a Sprague-Dawley (SD) reference strain. Neurally, we expected SHRs would show higher expression of cFos (a marker of neural activation) in the postsynaptic areas of the mesocorticolimbic dopamine pathway following an injection of cocaine compared to SD rats. Additionally, we sought to validate the hyperactive and inattentive phenotype of SHRs, expecting SHRs to have enhanced distance travelled in an open-field apparatus and fewer spontaneous alternations in a Y-maze test than SD rats.

Materials and Methods

Animals

This study utilised adolescent rats of approximately 5-7 weeks of age. Two strains of rats were chosen, one to represent the ADHD phenotype (spontaneously hypertensive rats [SHR]) and the other serving as a reference strain (Sprague-Dawley [SD]). SHR and SD subjects were bred in-house from male and female breeder rats originally obtained from Charles River Laboratory (Kingston, NY, USA). Animals were pair-housed with same-sex siblings in polycarbonate cages with wire top lids. Animals were maintained on a standard 12:12 hour light: dark cycle, with the dark phase beginning at 12 pm EDT/11 am EST. Hyperactivity and behavioural sensitisation tests were performed in the dark phase of the light-dark cycle as this is a period when the animals are more active. Inattention, however, was tested during the light

phase given that rats perform this test equivalently when tested during either the light or dark phase (Ghafouri et al., 2016); further, by moving this test to the light phase, both the inattention and the hyperactivity tests could be accomplished within the same testing day. The room housing the animal colony was temperature and humidity-controlled. Food (Labdiet 5001; F. Fisher & Son, Somerville, NJ, USA) and tap water were available *ad libitum*. Procedures performed on animals were in accordance with the *Guide for the Care and Use of Laboratory Animals 8th edition* (2011) and received prior approval from the Trinity College Animal Care and Use Committee (approval #: P68-23).

Drugs

Cocaine hydrochloride (C5776; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile physiological saline (0.9% NaCl) to a concentration of 10 mg/mL. Animals received intraperitoneal (IP) injections of either 10 mg/kg cocaine or sterile saline (1 mL/kg) during the behavioural sensitisation procedure. This 10 mg/kg cocaine dose was selected based on prior research performed on control rats to produce adequate behavioural sensitisation in peri-adolescent rats (Laviola et al., 1995) and confirmed in a pilot study.

ADHD Phenotype Validation

Overview

Subjects underwent an 8-day behavioural procedure as outlined in Figure 1. All rats were handled daily beginning at 4 weeks of age (postnatal day 28) before initiating this experimental procedure to acclimate them to being held by experimenters and to the intraperitoneal (IP) injection restraint posture. The two tests of ADHD phenotype, performed on day 1 and 7 of

overall testing, were the 8-minute spontaneous alternation test to measure the degree of inattention and the 10-minute open-field test, a measure of hyperactivity.

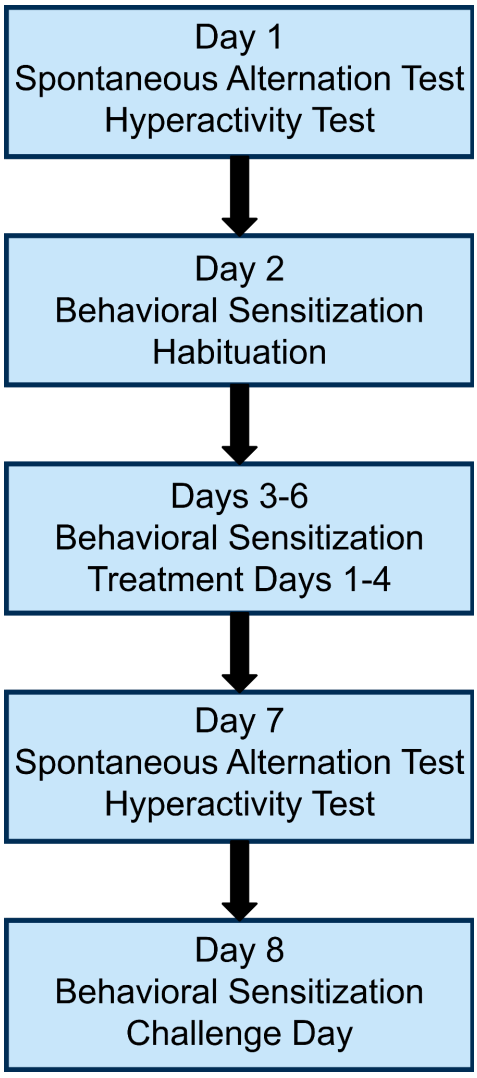

Figure 1. Timeline of the behavioural analysis procedures.

Spontaneous Alternations

On the morning of days 1 and 7 of the experimental procedure, rats were tested for spontaneous alternation behaviour using a Y-maze. This task was utilised to verify the

inattentiveness phenotype of ADHD (de la Peña et al., 2013; de la Peña, de la Peña, et al., 2015). The Y-maze apparatus consisted of three grey polycarbonate plastic arms (50L x 30W x 11H cm) stemming from a central platform at 120° angles (Maze Engineers, Skokie, IL, USA). Each rat was placed at the end of a randomly assigned arm (45.5L x 10.5W x 10.5H cm) of the apparatus and video recorded from above (Panasonic HC-V180 Full HD camera) as they moved freely through the three arms for the 8-minute testing duration. Testing was performed under white light, adjusted with the aid of a lux metre (LX1330B, Dr.meter) to be 15.7-16.1 lux at the level of the floor of each arm of the apparatus. At the conclusion of each test, animals were immediately removed from the apparatus and returned to their home cage. The Y-maze apparatus was thoroughly cleaned/sanitised via wipe down with Sani-Cloth Plus wipes (Q89072, Professional Disposables International, Inc., Woodcliffe Lake, NJ, USA) followed by 70% ethanol after each use.

Each animal's video recording was manually scored using BORIS (Friard & Gamba, 2016). An arm entry was defined as the entirety of the rat's head/torso crossing the threshold between the central platform and an arm. Spontaneous alternations were defined as any triplet sets of entries wherein the triplet incorporates three distinct arms. The percentage of spontaneous alternations was then calculated as the ratio of actual-to-possible spontaneous alternations, with the possible spontaneous alternations being defined as the total arm entries minus 2.

Distance Travelled

On days 1 and 7 of the experimental procedure, rats were tested for hyperactivity, a second key phenotype of ADHD. Rats were individually placed in the centre of a grey acrylic open-field chamber (60L x 60W x 40H cm; Maze Engineers, Skokie, IL, USA) and permitted to move freely for 10 minutes (de la Peña et al., 2013). The floor of this apparatus contained a 5x5

grid pattern (12 cm x 12 cm squares), with a central 4x4 grid region differentiated from the surround using a black wax marker. Testing was performed under dim light, adjusted to be 4.7-5.6 lux at the level of the floor of the apparatus, and maintained within a maximum deviancy of 0.2 lux between corners of the chamber. At the conclusion of each test, animals were immediately removed from the apparatus and returned to their home cage. The open-field apparatus was thoroughly cleaned/sanitised via wipe down with Sani-Cloth Plus wipes followed by 70% ethanol after each use. Testing was video recorded from above and the subsequent video was analysed for total distance travelled using ezTrack software for Windows (Pennington et al., 2021).

Behavioural and Neural Response to Cocaine

Overview

The rats underwent a 30-minute habituation period in the locomotor activity chamber on day 2. On days 3-6, the rats underwent the behavioural sensitisation protocol to record the sensitising effects of cocaine on behaviour. On day 7, the rats experienced a 48-hour forced abstinence period. On day 8, the rats underwent the challenge day of the behavioural sensitisation protocol, to determine the degree to which they had been sensitised to cocaine during the previous four treatment days. A subset of animals underwent immunohistochemistry procedures for the expression of cFos following the completion of the challenge day. These rats were euthanized approximately 60-70 minutes following the injection, perfused, and their brain tissue collected to be labelled for cFos.

Behavioural Sensitisation

Adolescent SHR and control rats underwent a behavioural sensitisation protocol (Laviola et al., 1995) as a behavioural indicator of the effects of cocaine on the rat's mesocorticolimbic

reward system. The apparatus (Kinder Scientific, Poway, CA, USA) consisted of a clear, acrylic chamber (43L x 21L x 20H cm) with a clear acrylic lid, situated within 2 metal sensing frames (upper and lower), each containing a grid of photo beams.

The behavioural sensitisation protocol consisted of a habituation day, four treatment days, and a challenge day (days 2, 3-6, and day 8 of the testing period, respectively). During the habituation day, the rats were individually placed into an assigned locomotor activity chamber and permitted to move freely for 30 minutes. On each subsequent treatment/challenge day, rats underwent a 30-minute habituation period in the chamber, were briefly removed in order to receive an injection of either saline (1 mL/kg) or cocaine (10 mg/kg), and then were returned to the chamber for a 60-minute test period. The Sal_Sal experimental treatment group received saline injections for the four treatment days and on the challenge day. The Sal_Coc treatment group received saline injections for the four treatment days and a cocaine injection on the challenge day. Lastly, the Coc_Coc treatment group received cocaine injections for the four treatment days and on the challenge day.

The pattern of breaks as the animal moved about the apparatus was recorded and subsequently analysed using Motor Monitor Software (Kinder Scientific). This software differentiated raw beam break data into ambulations and fine movements. Ambulations were defined as the rat clearing one photo beam and simultaneously breaking a new photo beam, indicative of the rat walking around within the apparatus. Fine movements were defined as all other photo beam breaks that did not meet the criteria for ambulations; these are often indicative of self-directed and/or repetitive movements such as grooming. The pattern of beam breaks allows the researcher to determine the rat's sensitisation to cocaine over repeated administration of cocaine or saline.

cFos Immunohistochemistry

Following the challenge-day behavioural sensitisation protocol, a subset of rats from Sal_Sal or Coc_Coc groups were randomly chosen to undergo labelling for cFos using immunohistochemistry. Rats were immediately removed from the locomotor activity chamber and euthanized using the open drop technique under a fume hood using isoflurane (W32965, Piramal, Bethlehem, PA, USA). They were then transcardially perfused with phosphate-buffered saline (PBS; pH 7.35-7.45) until the deep-coloured blood turned translucent/pale. This was followed by a perfusion of 4% paraformaldehyde in PBS for approximately 5 minutes, or until the neck stiffened. Brains were removed and post-fixed in 4% paraformaldehyde in PBS overnight, then submerged in 30% sucrose in PBS until the brain sank to the bottom of the vessel, indicating equilibrium. 35 μm coronal sections were taken using a cryostat. Sections of each animal's brain were collected from approximately +2.5 mm to +1.4 mm anterior to bregma as identified referencing a rat brain atlas (Paxinos & Watson, 2006); this range of collected tissue was inclusive of the prefrontal cortex and the nucleus accumbens. Sections were stored in cryoprotectant until labelling for cFos expression.

Labelling was performed at room temperature in free-floating tissue, and the steps are displayed in Table 1. Sections were initially rinsed in PBS and incubated in hydrogen peroxide (3% in PBS) for 15 minutes. This was performed to limit any endogenous peroxidase activity. Sections were rinsed again in PBS, then incubated in 3% normal goat serum (30017, Vector Laboratories, Newark, CA, USA) for 1 hour. Tissue was then incubated in 1:5000 rabbit anti-cFos primary antibody (AB190289, Abcam, Cambridge, UK) in 0.4% Triton-X (1002135494; Sigma-Aldrich, St. Louis, MO, USA) in PBS for 16-20 hours. After primary antibody incubation, tissue was rinsed in PBS, and subsequently incubated in 1:600 biotinylated

goat anti-rabbit secondary antibody (30014, Vector Laboratories, Newark, CA, USA) in 0.4% Triton-X in PBS for 1 hour. Sections were then rinsed in PBS and incubated for 1 hour in a solution of avidin-biotin complex (PK-6101, Vector Laboratories, Newark, CA, USA) in 0.4% Triton-X in PBS. Sections were rinsed with PBS and then in tris buffer (Trizma pre-set crystals [T7818; Sigma-Aldrich, St. Louis, MO, USA] in physiological saline). The tissue was then reacted in a 3,3'-diaminobenzidine tetrahydrochloride (DAB; 112090050; Acros Organics, Geel, Belgium) solution (2% DAB in tris buffer with hydrogen peroxide) for 15 minutes. Sections were then rinsed with tris buffer, followed by subsequent rinses in PBS. Tissues were mounted onto slides using a 1.4% bovine gelatin solution (G9391; Sigma-Aldrich, St. Louis, MO, USA) and dried. As described in Table 2, slides then underwent an ethanol series to further dehydrate the tissue, then cleared with CitriSolv (1601; Decon Labs, King of Prussia, PA, USA), and finally coverslipped using DPX mounting medium (13512; Electron Microscopy Sciences, Hatfield, PA, USA).

Step	**Solution**	**Duration**
1	PBS Rinse	10 x 5 minutes
2	3% hydrogen peroxide in PBS Incubation	15 minutes
3	PBS Rinse	10 x 5 minutes
4	3% normal goat serum in PBS Incubation	1 hour
5	Rabbit anti-cfos antibody in 0.4% Triton-X PBS Incubation	16-20 hours

6	PBS Rinse	10 x 5 minutes
7	Biotinylated Goat Anti-Rabbit antibody in 0.4% Triton-X PBS Incubation	1 hour
8	PBS Rinse	10 x 5 minutes
9	Avidin-biotin complex in 0.4% Triton-X PBS Incubation	1 hour
10	PBS Rinse	3 x 5 minutes
11	Tris Buffer Rinse	3 x 5 minutes
12	DAB in Tris Buffer Incubation	15 minutes
13	Tris Buffer Rinse	3 x 5 minutes
14	PBS Rinse	5 x 5 minutes
15	Bovine gelatin	Mount on slide

Table 1. Immunohistochemical procedure for the staining of cFos.

Step	Solution	Duration
1	Deionized Water	1 minute
2	70% Ethanol	10 minutes
3	95% Ethanol	10 minutes
4	100% Ethanol	10 minutes
5	100% Ethanol	10 minutes
6	Citrisolv	5 minutes
7	Citrisolv	5 minutes

Table 2. Dehydrating and clearing process.

Slides were viewed using a Nikon Eclipse E600 light microscope at 10X magnification and images were captured using Nikon DS-Ri2 digital camera and Nikon NIS Elements software. Originally, the nucleus accumbens core (NAcCo) and shell (NAcSh), the caudate putamen (CPu), and the infralimbic (IL), prelimbic (PrL), and cingulate (Cg) cortices were planned to be evaluated for expression of cFos. Rectangular counting domains (487 x 366 μm) were fitted to both hemispheres of each brain area, and cells positive for cFos were identified by brown soma staining, as displayed in Figure 2. Images were cropped using Inkscape software to the size of the counting domain. Due to time constraints, the cell counter plug-in for ImageJ was

not utilised to count the number of cFos-positive cells and data was only assessed qualitatively; furthermore, this qualitative assessment was strictly limited to just the NAcCo of female rats. These cell counts were planned to be averaged across the hemispheres and subsequently divided by the area of the counting domain, resulting in cell densities for each brain area assessed.

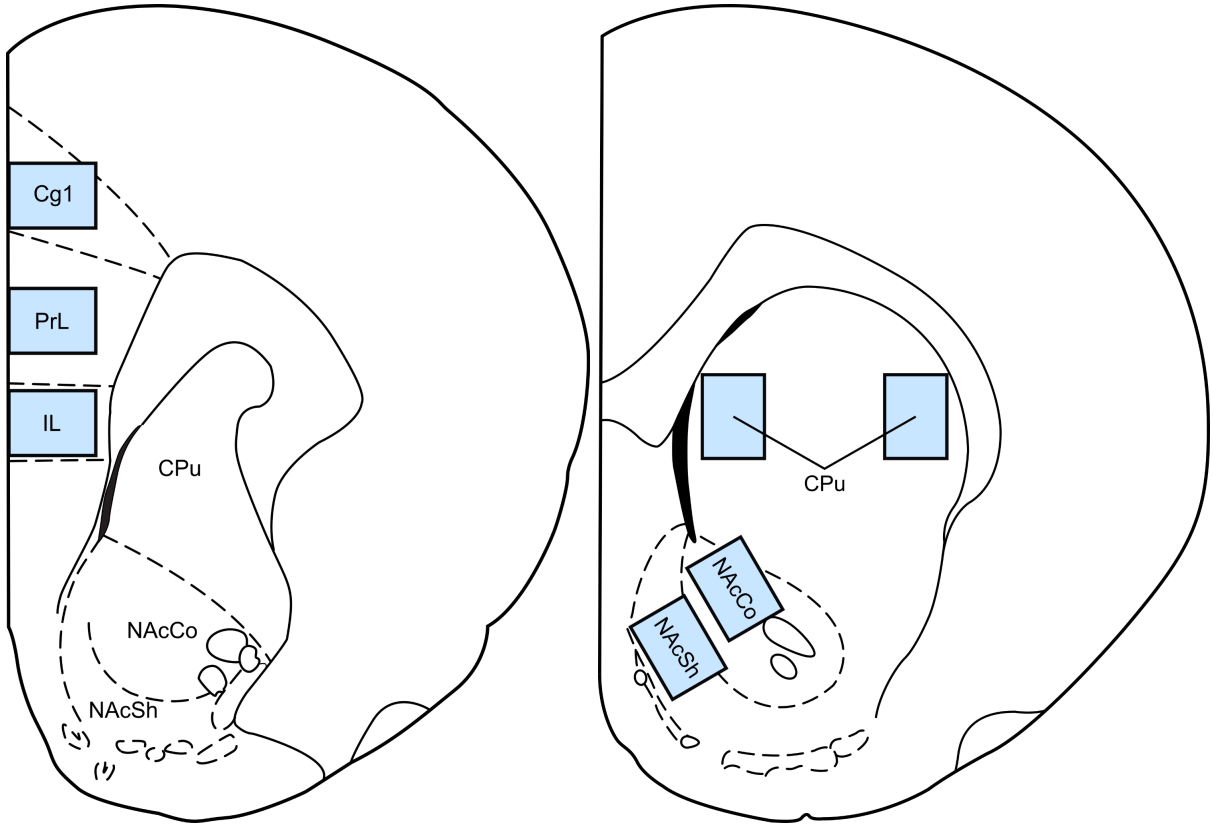

Figure 2. Example counting regions (487 x 366 µm) in brain areas bregma +2.20 (left) and bregma +1.20 (right; modified from Paxinos & Watson, 2006).

Statistical Data Analyses

All data were analysed using JASP for Windows (JASP Team, 2023). Data descriptives were initially assessed for skewness, kurtosis, and normality (Shapiro-Wilk test) to determine if

the assumptions of parametric statistical tests were met. In the case of a violation of these assumptions, outliers identified by JASP were removed from the data set. A single outlier was removed from the hyperactivity posttest analyses, resulting in final sample sizes of n = 9-12 per treatment group in all statistical analyses.

Behavioural assessments made prior to any drug injections (i.e., pretest percent successful spontaneous alternations (Y-maze) and total distance travelled (open-field)) were initially assessed for an effect of treatment group (Sal_Sal/Sal_Coc/Coc_Coc) using a factorial ANOVA. This single-factor analysis was conducted in order to confirm that there were no pre-existing differences across assigned treatment groups for these measures, prior to any treatment administration. These measures were subsequently re-analysed using 2x2 ANOVAs, with strain (SD/SHR) and sex (female/male) as independent factors. Posttest behavioural analyses (Y-maze and open-field) and the number of ambulations and fine movements (challenge day) were assessed using 2x2x3 ANOVAs with strain (SD/SHR), sex (female/male), and treatment (Sal_Sal/Sal_Coc/Coc_Coc) as independent factors. Initially, the density of cells expressing cFos in each brain area was assessed using 2x2x2 factorial ANOVA with strain (SD/SHR), sex (female/male), and treatment (Sal_Sal/Coc_Coc) as independent factors were planned to be performed. However, due to time constraints, the cells expressing cFos were only qualitatively assessed and focused on females. All significant interactions were followed up on using Tukey's Post Hoc analyses. For all analyses, results were deemed statistically significant if $p < 0.05$.

Results

ADHD Phenotype Validation

Inattentive Phenotype

The data yielded from the spontaneous alternation pretest were analysed using a factorial ANOVA to determine if SHRs differ from SD rats in the severity of inattention compared to SD rats. Initial assessment of treatment assignment revealed a non-significant main effect of treatment, $F(2,127) = 1.603$, $p = 0.205$, indicating no initial differences in hyperactivity among the assigned groups. The subsequent factorial ANOVA (excluding treatment group) revealed no significant main effects for either strain, $F(1, 126) = 2.629$, $p = 0.107$, or sex, $F(1, 126) = 0.083$, $p = 0.774$, on the percentage of successful spontaneous alternations (Figure 3). To evaluate if there was any effect of time/experience on this measure, a factorial ANOVA was performed to elucidate the effects of drug treatment on the inattentive phenotype in spontaneous alternation post-test data. It was found that there was no significant effect of strain $F(1,89) = 1.317$, $p = 0.254$, sex $F(1,89) = 0.024$, $p = 0.876$, nor treatment group $F(2,89) = 0.686$, $p = 0.506$, upon the successful spontaneous alternation percentages.

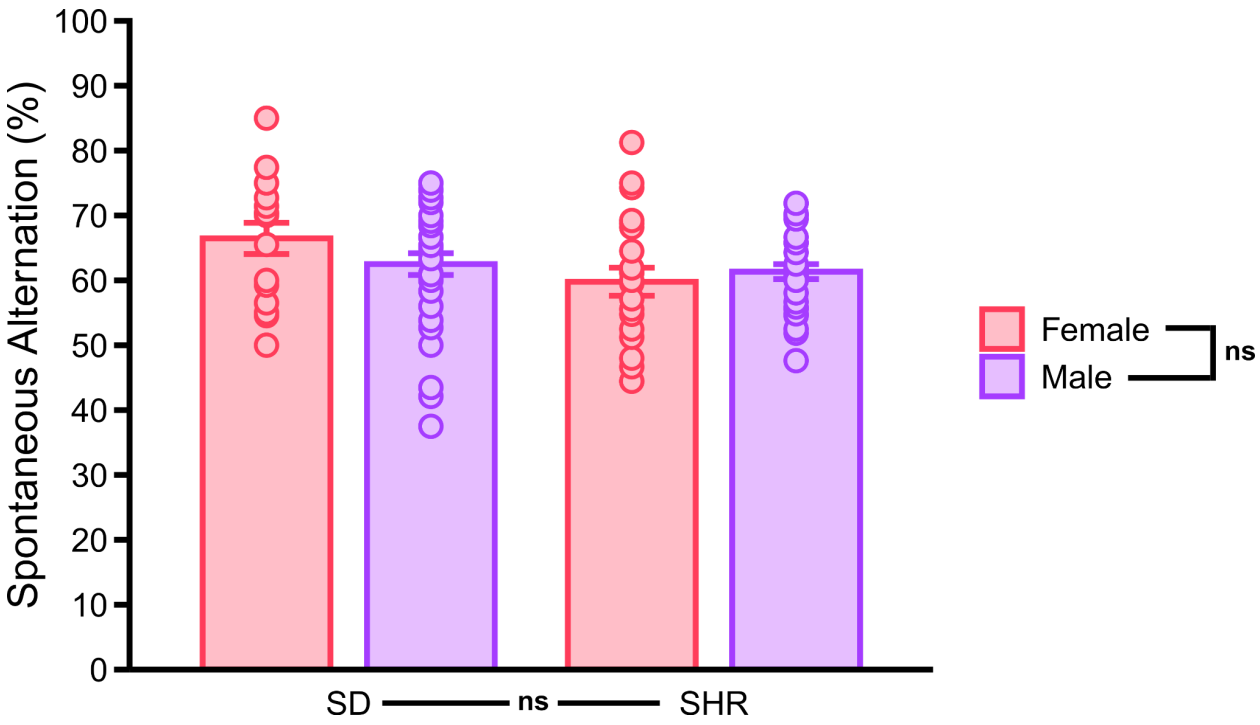

Figure 3. Mean (+/- SEM) successful spontaneous alternation percentage pretest scores for SHR and SD rats. ns = not significant (main effects of sex/strain).

Hyperactive Phenotype

The data yielded from the hyperactivity pretest were analysed using a factorial ANOVA to determine if SHRs differ from SD rats in the severity of hyperactivity. Initial assessment of treatment assignment revealed a non-significant main effect of treatment, $F(2,128) = 0.855$, $p = 0.428$, indicating no initial differences in hyperactivity among the assigned groups. The subsequent ANOVA found a significant effect of strain on distance travelled, $F(1,127) = 11.520$, $p < 0.001$, with an increased distance travelled for SHRs compared to SD rats (Figure 4). A

significant effect of sex F(1,127) = 6.690, p = 0.011, with an increased distance travelled for females compared to males was also found.

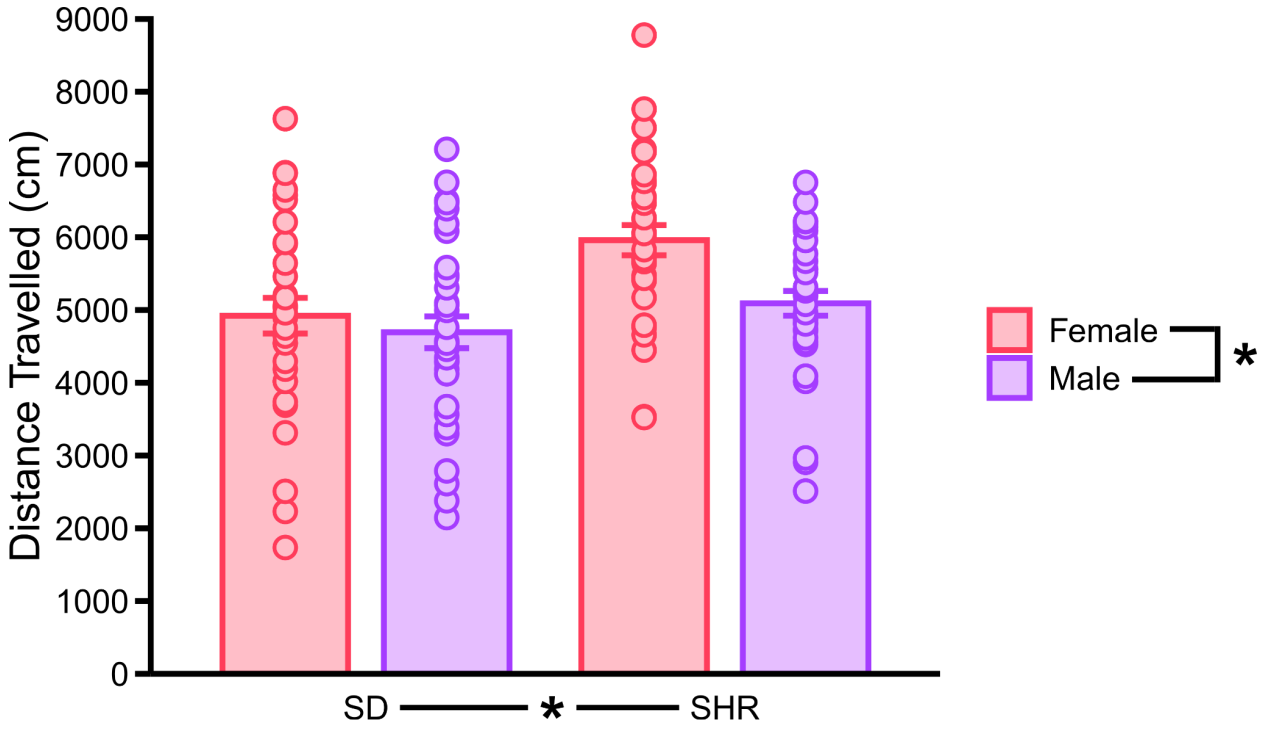

Figure 4. Mean (+/- SEM) distance travelled in the pretest for male and female SHR and SD rats. *p<0.05, main effects of strain and sex on distance travelled.

To evaluate if these strain and sex effects persisted over time, a factorial ANOVA was performed to elucidate the effects of drug treatment on the hyperactive phenotype in hyperactivity post-test data. One outlier was identified in the male SHR Sal_Sal group, and was subsequently removed from the statistical analyses. It was found that there were no significant main effects of strain F(1, 118) = 0.275, p = 0.601, sex F(1, 118) = 0.287, p = 0.593, nor

treatment group $F(2,118) = 0.006$, $p = 0.994$, upon distance travelled. However, a significant interaction effect among sex and strain was found, $F(1, 118) = 6.971$, $p = 0.009$. To further examine this interaction, specific pairwise comparisons were conducted contrasting strains within each level of sex, using Tukey's HSD to correct for multiple comparisons. This analysis revealed no significant differences among any of the pairwise comparisons.

Behavioural and Neural Response to Cocaine

Behavioural Sensitization

A factorial ANOVA was utilised to examine the effect of experimental group on ambulatory activity and fine movements during the pre-injection phase of challenge day. There was a significant main effect of strain on the number of ambulations, $F(1,119) = 60.560$, $p < 0.001$ (Figure 5). Specifically, ambulations for SHRs were increased compared to SD rats, further validating the hyperactive phenotype in SHRs. Additionally, there was a significant main effect of sex $F(1,119) = 13.006$, $p < 0.001$, with an increased number of ambulations for females compared to males. There was, however, no significant effect of treatment group on pre-injection ambulations ($F(2,119) = 0.646$, $p = 0.526$. In contrast, there were no significant effects of strain, $F(1,119) = 1.610$, $p = 0.207$, sex, $F(1,119) = 0.814$, $p = 0.369$, or treatment group, $F(2,119) = 0.487$, $p = 0.616$, on fine movements made during the pre-injection period (Figure 6).

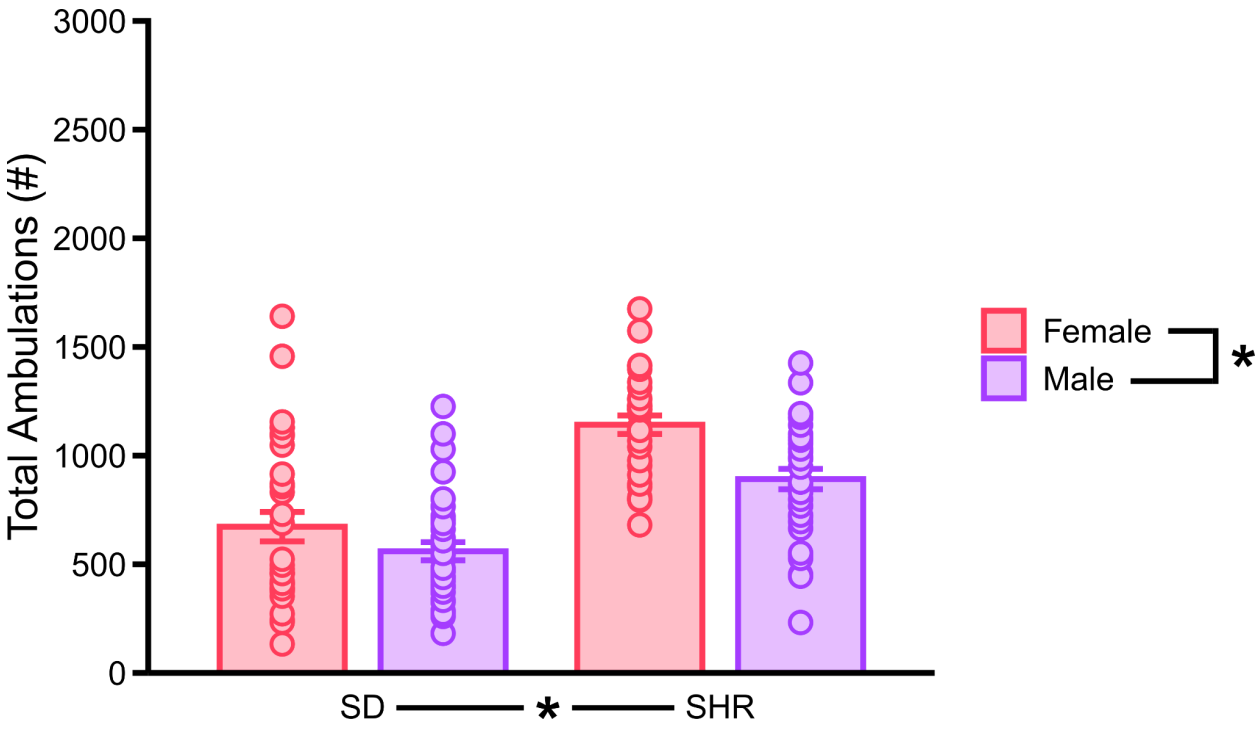

Figure 5. Mean (+/- SEM) ambulations made during the challenge day pre-injection period for male and female SHR and SD animals. *p<0.05, main effects of strain and sex on challenge day ambulations.

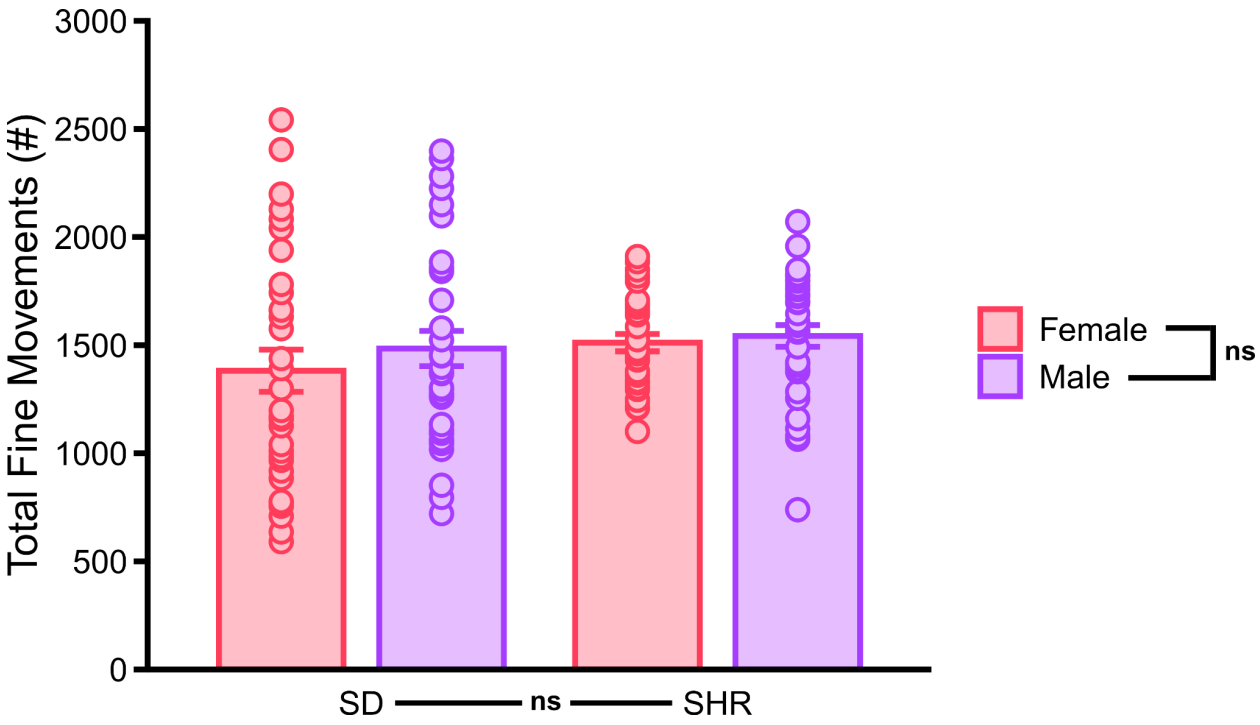

Figure 6. Mean (+/- SEM) fine movements made during the challenge day pre-injection period for male and female SHR and SD animals. ns = not significant (main effects of sex/strain).

Post-injection data from the challenge day were analysed using a factorial ANOVA, in order to determine differences in the ambulatory response to cocaine across experimental conditions. Similar to the pre-injection findings, there was a significant main effect of strain on ambulations, $F(1,119) = 10.636$, $p = 0.001$, with increased activity again observed in SHRs compared to SD rats. A significant main effect of sex, $F(1,119) = 27.819$, $p < 0.001$, was also observed, with increased ambulations for females compared to males. Additionally, a significant main effect of treatment group, $F(2,119) = 72.031$, $p < 0.001$, was found, with Coc_Coc animals responding the most robustly to the injection on challenge day, and Sal_Sal animals responding the least. This pattern of results is closely mirrored in the analysis of fine movements performed.

Significant main effects of sex, $F(1,119) = 6.688$, $p = 0.011$, and treatment group, $F(2,119) = 105.515$, $p < 0.001$, were observed in the same direction as the ambulatory results. However, the effect of strain was not statistically significant for this measure, $F(1,119) = 3.758$, $p = 0.055$.

Importantly, the effects of treatment group and strain were inconsistent across the sexes for both measures, as evidenced by a significant three-way interaction effect in both ambulatory response, $F(2,119) = 3.371$, $p = 0.038$, and in fine movements, $F(2,119) = 8.507$, $p < 0.001$. To further examine these interactions, specific pairwise comparisons were conducted contrasting treatment groups within each level of sex/strain using Tukey's HSD to correct for multiple comparisons. In both the ambulatory (Figure 7) and the fine movements (Figure 8) data, no significant difference was found among female SD Sal_Coc and Coc_Coc, indicating a failure to sensitise to 10 mg/kg cocaine in this behavioural sensitisation protocol. However, among female SHRs, Coc_Coc responded more than the Sal_Coc treatment groups, indicating successful sensitisation to cocaine. The acute response to cocaine was intact in both SD and SHR females, as evidenced by significantly greater ambulatory and fine movement responses in Sal_Coc vs. Sal_Sal in both strains.

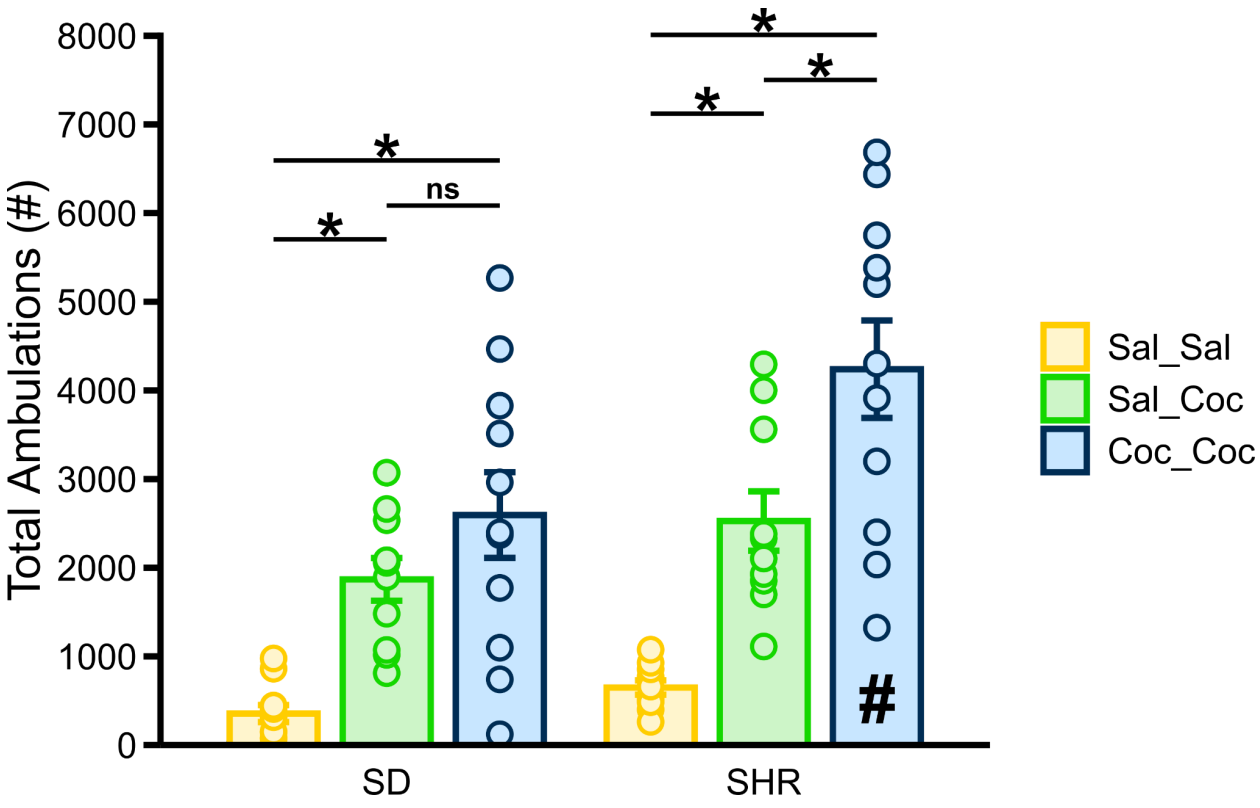

Figure 7. Mean (+/- SEM) ambulations on challenge day post-injection period for female SHR and SD animals. Sal_Sal animals were injected with saline during treatment days and received saline during challenge day. Sal_Coc animals were injected with saline during treatment days and received cocaine during challenge day. Coc_Coc animals were injected with cocaine during treatment days and received cocaine during challenge day. *$p < 0.05$, and ns = non-significant, Tukey HSD-corrected pairwise comparisons of treatment groups within each strain. #$p<0.05$, Tukey HSD-corrected pairwise comparison of strains within specific treatment groups.

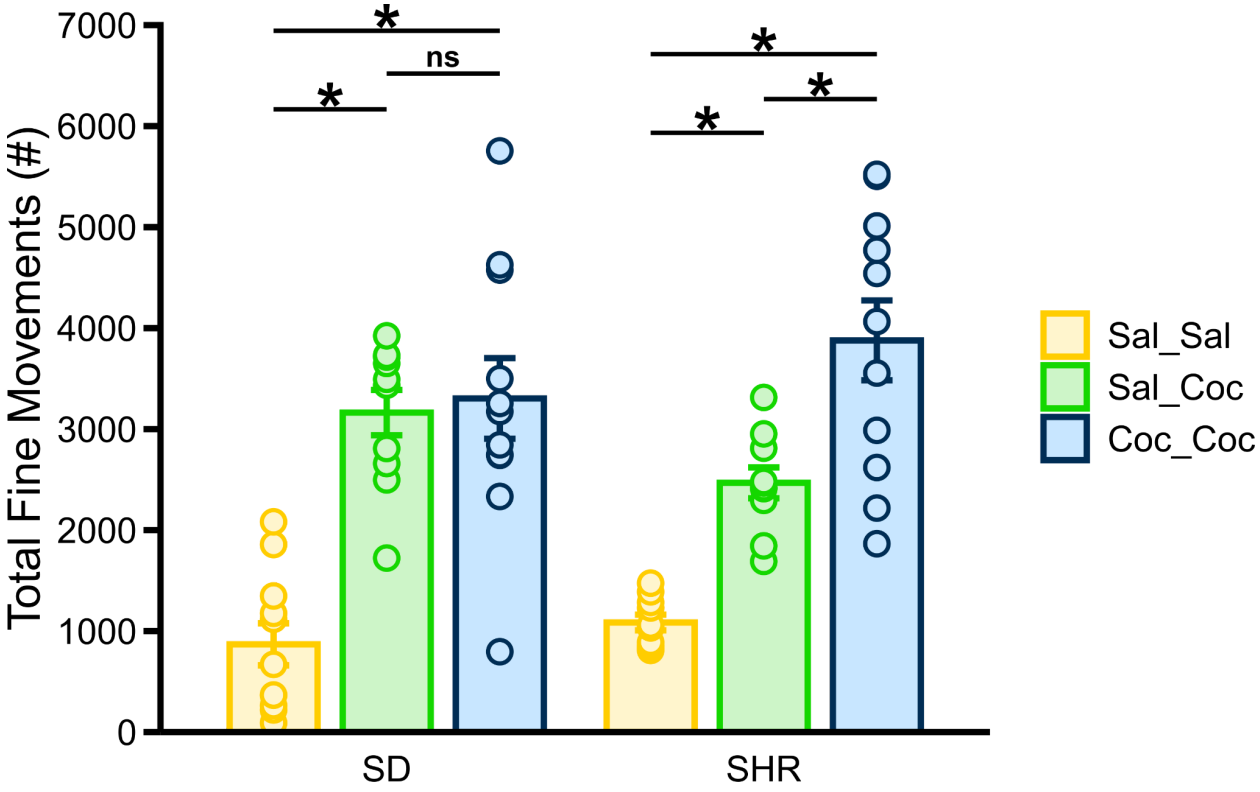

Figure 8. Mean (+/- SEM) fine movements on challenge day post-injection period for female SHR and SD animals. Sal_Sal animals were injected with saline during treatment days and received saline during challenge day. Sal_Coc animals were injected with saline during treatment days and received cocaine during challenge day. Coc_Coc animals were injected with cocaine during treatment days and received cocaine during challenge day. *$p < 0.05$, and ns = non-significant, Tukey HSD-corrected pairwise comparisons of treatment groups within each strain.

A contrasting pattern of results was observed in male ambulations (Figure 9) and fine movements (Figure 10). Unlike the female SD rats, male SD rats showed significant differences among the Sal_Coc and Coc_Coc treatment groups, indicating sensitisation to cocaine. However,

in male SHRs, the Sal_Coc and Coc_Coc treatment groups were not found to be significantly different, indicating a failure to sensitise to 10 mg/kg cocaine in this behavioural sensitisation protocol. Furthermore, a lack of significant difference in the Sal_Coc vs. Sal_Sal treatment groups' ambulatory and fine movement response in SHR males indicates the acute response to cocaine did not trigger a strong behavioural response.

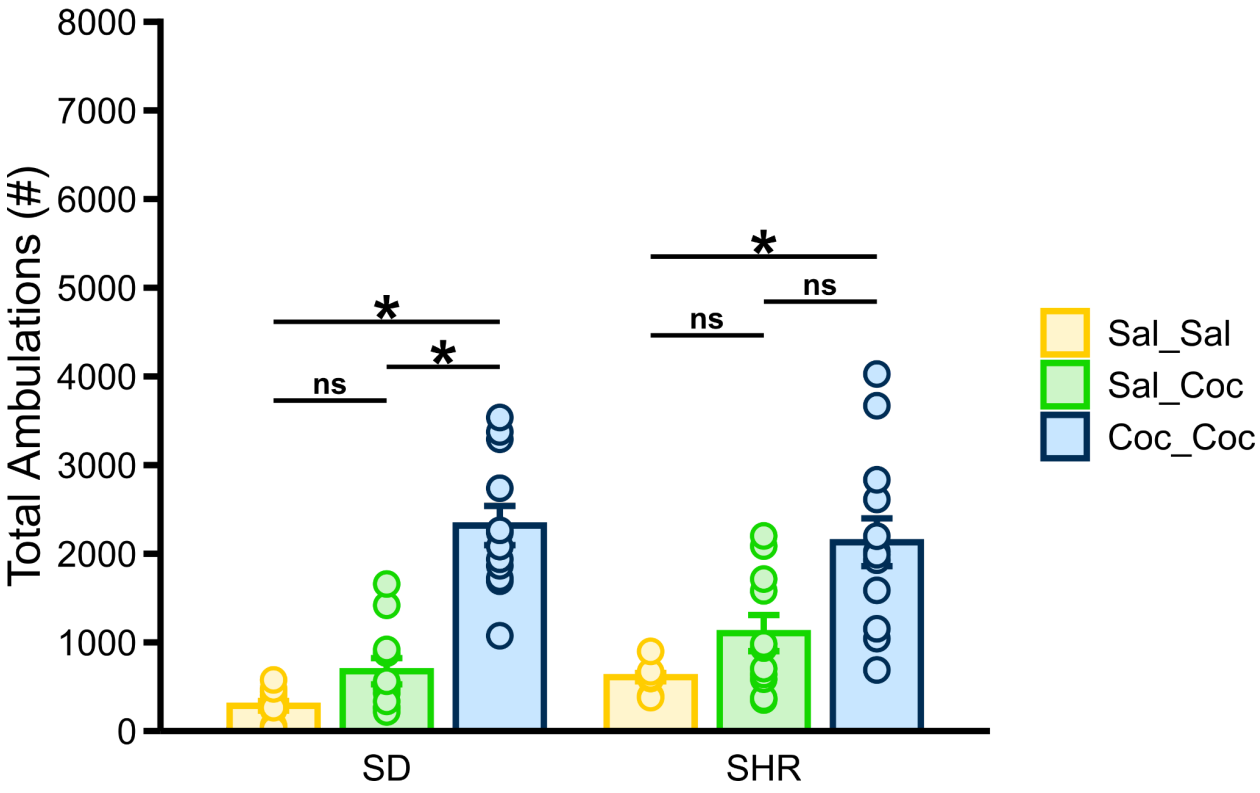

Figure 9. Mean (+/- SEM) ambulations on challenge day post-injection period for male SHR and SD animals. Sal_Sal animals were injected with saline during treatment days and received saline during challenge day. Sal_Coc animals were injected with saline during treatment days and received cocaine during challenge day. Coc_Coc animals were injected with cocaine during

treatment days and received cocaine during challenge day. *p < 0.05, and ns = non-significant, Tukey HSD-corrected pairwise comparisons of treatment groups within each strain.

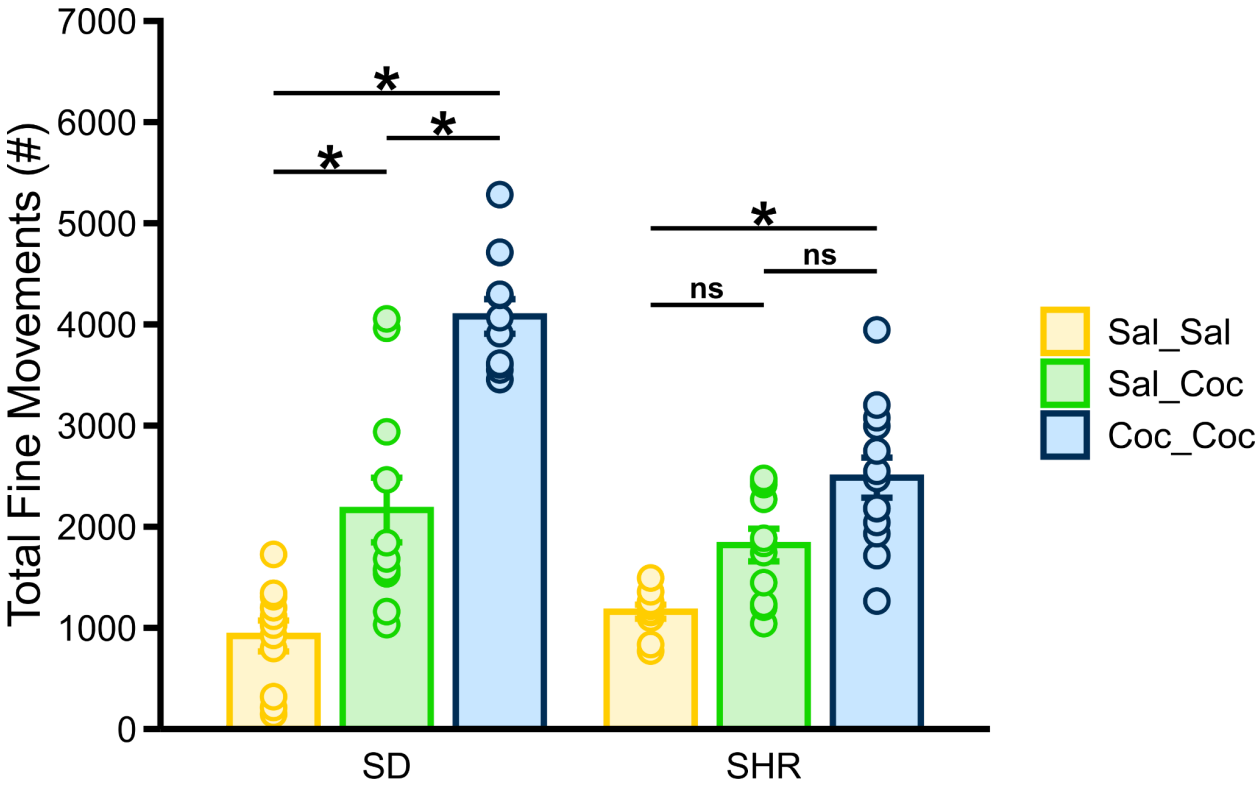

Figure 10. Mean (+/- SEM) fine movements on challenge day post-injection period for male SHR and SD animals. Sal_Sal animals were injected with saline during treatment days and received saline during challenge day. Sal_Coc animals were injected with saline during treatment days and received cocaine during challenge day. Coc_Coc animals were injected with cocaine during treatment days and received cocaine during challenge day. *p < 0.05, and ns = non-significant, Tukey HSD-corrected pairwise comparisons of treatment groups within each strain.

In addition to examining the effect of treatment group within each strain (described above), the effect of strain within each treatment group was examined to determine if the enhanced sensitisation in SHR females could be attributed to differential strain responses to specific cocaine treatment patterns. Specific pairwise comparisons were conducted contrasting treatment groups within each level of sex/strain, using Tukey's HSD to correct for multiple comparisons. In comparing the SHR and SD strains within the Coc_Coc treatment group, significant differences were found among female SD rats and female SHRs, with female SHRs performing greater ambulations than female SD rats (Figure 7). This pattern was not observed among the fine movement data (Figure 8). This strain difference was also not found among male SD rats and male SHRs in the Coc_Coc treatment group ambulations (Figure 9) or fine movement data in either sex (Figure 10). Furthermore, no significant differences in strain ambulations or fine movements were found among the Sal_Sal groups nor the Sal_Coc groups of either sex. The significant effects indicate sex differences in the matter in which the SHRs and SD rats are behaviourally sensitising to cocaine and further support the differential strain responses to cocaine treatment patterns.

cFos Immunohistochemistry

Although cells expressing cFos have yet to be quantified, a limited qualitative analysis was performed to elucidate the effect of the experimental group in females. SHR Coc_Coc females appear to have greater expression of cFos in the left nucleus accumbens core than SHR Sal_Sal females (Figure 11). This preliminary finding supports the expected finding of increased cFos expression following an injection of cocaine.

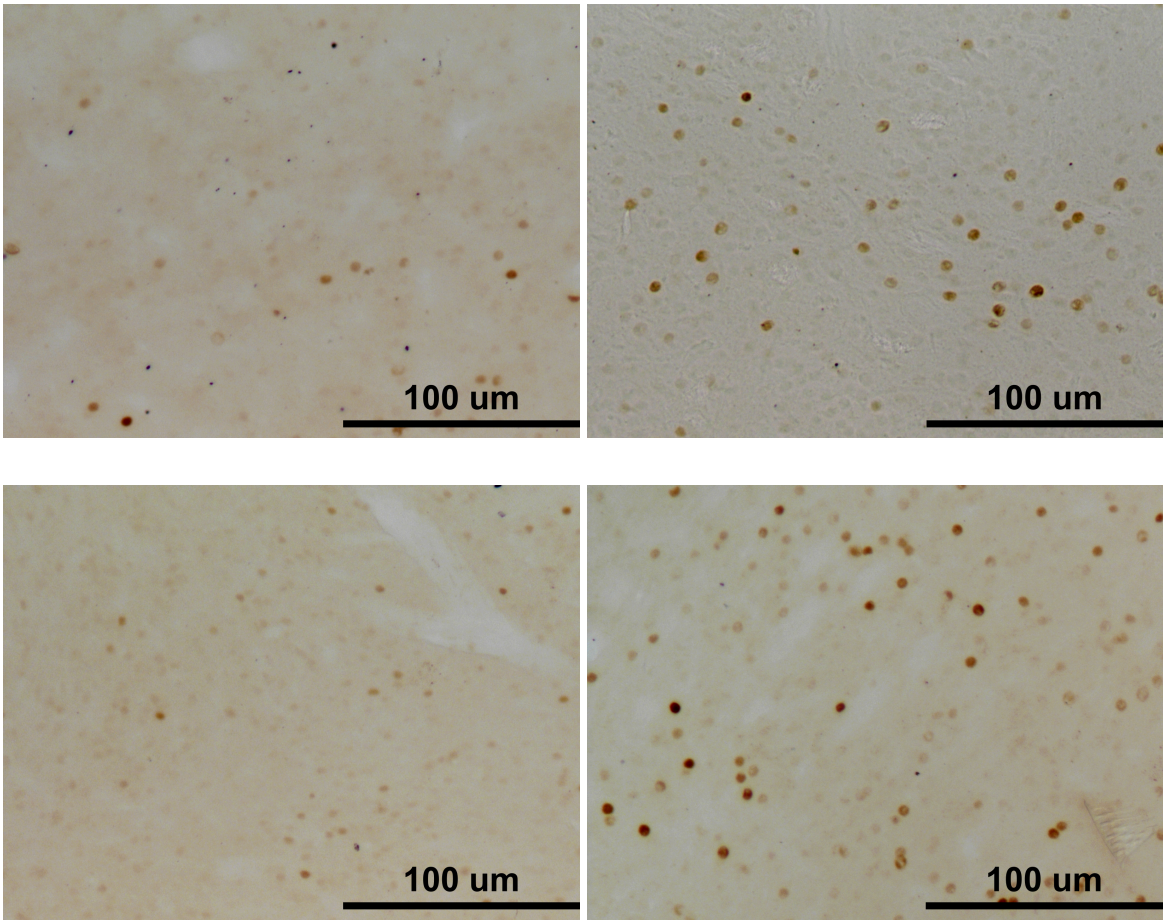

Figure 11. Enhanced, representative subsections of images taken of the left nucleus accumbens core (244 x 183 μm) of female rats following cFos immunohistochemistry protocol. Dark brown staining is indicative of a cell expressing cFos. Female SD rat that received only saline injections (top left), female SD rat that received only cocaine injections (top right), female SHR that received only saline injections (bottom left) and female SHR that received only cocaine injections (bottom right).

Discussion

In this study, we were able to replicate the hyperactive, but not inattentive, phenotype in SHRs. The replication of these characteristics of ADHD was important to validating the SHR as a rodent model of ADHD, and thus we were partially successful in this venture. Furthermore, we demonstrated an interesting sex and strain difference in the behavioural response to cocaine. By comparing the acute response to 10 mg/kg cocaine to the sensitised effects of this dose (i.e., Sal_Coc vs. Coc_Coc treatment groups), we observed female SHRs sensitised more robustly than the female SD rats. In contrast, we observed the opposite pattern of results in males, with male SHRs demonstrating decreased locomotor sensitisation to this dose of cocaine relative to the reference animals. Furthermore, preliminary qualitative analysis of neural responses to cocaine on challenge day appears to mirror the behavioural findings in female rats. Specifically, increased expression of cFos was seen in female SHRs relative to the reference SD rats. Overall, we validated the SHR as a rodent model of the hyperactive subtype of ADHD and demonstrated a novel altered neurobehavioural response to cocaine in the SHR strain that differed across the sexes. These findings indicate that adolescent females with ADHD may be at an increased risk of developing substance use disorders to psychostimulant drugs like cocaine.

One goal of this study was to replicate previous studies evaluating the spontaneously hypertensive rats (SHRs) inattentive and hyperactive phenotype, in order to determine if this strain is an adequate representation of ADHD. A previous study in adolescent male SHRs demonstrated increased distance travelled relative to a reference strain (Tsai et al., 2017). These results were replicated in the current study, as SHRs exhibited a higher moved distance in the open-field apparatus compared to the SD reference animals, thus validating the hyperactivity in SHRs. Additionally, the hyperactive phenotype was also observed during the pre-injection phase

on the challenge day of the behavioural sensitization protocol, as evidenced by significantly higher ambulations in SHRs compared to reference animals during this test. Together, the increased distance travelled and the increased ambulations observed in the SHR strain indicate a hyperactive phenotype in these rats.

In a previous study, adolescent male SHRs demonstrated impaired spontaneous alternation percentages in a Y-maze test relative to a reference strain, indicating the inattentive phenotype in this strain (de la Peña, Bang, et al., 2015). The results of the current study, however, failed to support the SHR strain as expressing the inattentive phenotype of ADHD. One possible explanation is the effect of lighting on the percent spontaneous alternation. The other behavioural measures, both hyperactivity and behavioural sensitisation to cocaine, were performed under dark light, whereas the spontaneous alternation test was performed under white light. This may impact the results as rats are traditionally nocturnal and therefore performing testing under white light may have altered their traditional exploratory behaviour. However, this proposed explanation is contradicted by a previous study that performed Y-maze testing in adolescent male reference rats across both light conditions, which found no effects of lighting conditions on spontaneous alternation behaviour (Ghafouri et al., 2016).

Alternatively, it may be the case that the SHRs have experienced sufficient genetic drift such that they are no longer a valid model of the inattentive phenotype. However, if this were the case, this drift would appear to be exceptionally limited, given the other aspects of the SHR phenotype appear to be fully intact in the rats evaluated here. As previously discussed, this study successfully replicated the hyperactivity phenotype in SHRs across several different behavioural tests. Hyperactivity remains an important component of ADHD in humans, with the predominantly hyperactive-impulsive subtype of ADHD being of greater prevalence than the

combined hyperactive/inattentive subtype (Ayano et al., 2023). Hyperactivity is therefore sufficient validation of the ADHD phenotype for the purpose of this study. Additionally, we confirmed the rats used in this study also expressed several previously reported features of the SHR phenotype. SHRs typically display a decreased anxiety profile, as indicated by increased distance travelled and time spent in the centre of an open-field apparatus (Mc Fie et al., 2012; Ramos et al., 1997). In the present study, SHRs spent a significantly greater amount of time in the centre of the apparatus (data not shown), further indicating the SHR strain performed in an anticipated manner for a rodent model of ADHD. Additionally, the SHRs demonstrated a reduced weight gain profile compared to the SD reference strain (data not shown) in excellent agreement with what has been previously described (Ferguson et al., 2003).

Another possible explanation for the spontaneous alternation test results in the current study is an unexpected inattentive phenotype in the reference strain. SD rats were anticipated to perform greater spontaneous alternations but instead performed much closer to the past, published SHR levels (Liet et al., 2015; Ramanathan et al., 2010). Though the exact mean percent spontaneous alternations were not reported, previous studies in male SD rats depict an approximate 70% spontaneous alternation, whereas SHRs have previously performed successful spontaneous alternations in approximately 60% of the test (de la Peña, Bang, et al., 2015). The present study reported an approximate 60% spontaneous alternation in SD rats of both sexes, therefore indicating a potential discrepancy in reference strain performance that may be underlying the failure to observe significant differences. This raises the question of whether the SD rats used in this study are the best reference for SHR phenotype performance and/or whether the Y-maze test for spontaneous alternations is the most sensitive test for evaluating the inattentive phenotype in SHRs when compared to SD rats. In the future, an alternative measure

of inattentiveness is required to determine whether it is the SD reference or the test itself that is causing this pattern of results. Modified Y-maze tests have been utilised to assess working memory in SHRs; one such technique is temporarily confining the animal to two of the arms, then allowing access to all three and measuring the amount of time spent in the novel arm as an alternative indicator of spatial working memory (Simchon-Tenenbaum et al., 2015). Further research using this modified Y-maze test may be able to determine whether it is the SHR model of ADHD causing these complications or the test itself.

The animals were re-assessed for their ADHD phenotype following the four treatment days of the behavioural sensitisation protocol. Analysis of this posttest data failed to demonstrate the hyperactive phenotype at this time point. One such reason is the effect of retesting the rats, with repeated exposure to the testing apparatus influencing the rodents' pattern of behaviour. This is supported by previous literature demonstrating the distance travelled by SD rats decreased with repeated exposure to the open-field apparatus (Valle, 1971). This theory is further supported by the pre-injection phase on the challenge day of the sensitisation protocol, where during this period, the hyperactive phenotype was demonstrated in both sexes, mirroring the patterns observed on the open-field apparatus pretest. This suggests that the hyperactive phenotype is intact, but is not represented due to the repeated exposure to the apparatus in this test. Additionally, upon the posttest day, these rats were in the midst of the 48-hour abstinence period that is commonly performed in the behavioural sensitisation protocol. During this time, the rats may have been experiencing withdrawals from cocaine that would have led to a decrease in locomotor activity. Indeed, withdrawal following extended cocaine exposure has been demonstrated to decrease locomotor activity (Calipari et al., 2013). However, this is again refuted by the locomotor activity represented in the pre-injection phase of challenge day

demonstrating a strain difference. Therefore, this is not a likely explanation for the lack of hyperactive phenotype present in SHRs in the open-field posttest. Taken together, we fail to reach a meaningful conclusion on the effects of cocaine exposure on the ADHD phenotype in SHRs.

The novel findings of the behavioural sensitisation protocol reveal interesting sex and strain differences. Previous studies assessing the effects of stimulants in adolescent SHRs relative to controls have focused on males, and have failed to find any significant strain effects. One such study evaluated the effects of methamphetamine in adolescent SHRs on conditioned place preference (a measure of motivation for a stimulus) and failed to find significant strain differences (de la Peña et al., 2010). Methamphetamine and cocaine differ in their effects on glutamate in the ventral tegmental area and the substantia nigra, wherein acute methamphetamine decreased glutamate overflow but acute administration of cocaine increased it (Zhang et al., 2001). This points to a difference in neural response to these psychostimulants that could lead to an altered behavioural response. Furthermore, the conditioned place preference test evaluates an animal's ability to associate the rewarding effects of cocaine with a particular chamber in an apparatus. The degree of conditioned place preference, however, has not been found to be correlationally tied to behavioural sensitisation severity in SD rats (Seymour & Wagner, 2008). Thus, these behavioural measures of response to a drug of abuse may not parallel one another.

There is a possibility that the observed differences in behavioural response may instead arise from the differences in developmental stage across the two strains. The present study opted to follow previous literature evaluating adolescent SHRs by using age-matched control and experimental rats; as such, one potential explanation for the observed behavioural differences is

the precise developmental stage of the SHR relative to its reference strain. The SHR strain has been shown to be developmentally delayed when compared to SD rats, including delayed eye-opening and the aforementioned decreased body weight (Ferguson et al., 2003). Importantly, SHRs have also shown alterations in the onset of puberty, including delayed vaginal opening (Hashimoto & Kimura, 1989), which may alter the sex steroid hormones present in SHRs vs. SD rats. If the SHRs are developmentally delayed, as the literature supports, then SHRs would likely have less estradiol and testosterone than the reference strain at this period in adolescence. Estradiol has been previously demonstrated to alter the development of addiction to cocaine, with ovariectomized female rats that received estradiol replacement treatment self-administering significantly greater cocaine infusions than ovariectomized rats that lacked estradiol replacement (Jackson et al., 2006). Furthermore, greater locomotor responses to cocaine have been observed in female SD rats as they transition from prepubertal, to pubertal, and then into adulthood, (Laviola et al., 1995), further implicating differential availability of estradiol as a potential factor driving observed strain differences within females. Testosterone has also been implicated in the development of addiction, with pretreatment of testosterone significantly decreasing ambulatory behaviour following cocaine administration in adolescent male rats (Minerly et al., 2010). If it were the case that estradiol is modulating the behavioural sensitising effects of cocaine in females, one would anticipate the SD rats, being further along in puberty and thus having closer to adult levels of estradiol, would exert greater sensitisation relative to the developmentally delayed SHRs. Additionally, one would anticipate the raised testosterone levels in the SD, assuming they are further developed at this time, to have a protective effect on the development of sensitisation to cocaine. In this study, the opposite pattern of results was observed, thus refuting the developmental delay and consequent impact on pubertal hormone levels as distinct

variables modulating the altered behavioural response in SHRs. However, it may be beneficial to compare developmentally matched and chronologically matched groups of SHR and SD rats in future studies to account for potential differences in estradiol and testosterone levels due to altered pubertal trajectories.

Although pubertal changes in gonadal steroid hormone levels are unlikely to explain the pattern of sex/strain effects observed in the present study, differential hormone exposure earlier during development may still have some impact. The effects of prenatal hormone exposure have a significant impact on the rats' sexual and reproductive development later in life. If it were the case that the organisational effects of increased prenatal androgen exposure were to alter the development of the reproductive system in rats, this could also alter the developmental trajectory of the reward pathway and thus lead to differences in the SD/SHR. This has been demonstrated in previous studies in mice that found that prenatal exposure to androgens increased adult alcohol consumption in females whereas androgen receptor inhibition decreased male alcohol consumption (Huber et al., 2018). This ties a link between prenatal androgen exposure and subsequent addictive behaviours in adulthood. If it were the case that female SHRs had increased prenatal androgen exposure than the reference strain, the observed increase in locomotor response to cocaine would align with these prior results. Contrastingly, if male SHRs were exposed to lowered prenatal androgens, this would be expected to decrease sensitisation in SHR males in the manner we observed. No studies have directly assessed the prenatal androgen exposure of the SHR and how it may differ from the reference strain, thus it is inconclusive whether the prenatal hormone exposure may be leading to altered behaviour. Thus, to evaluate this indirectly, we look to sex differences in early life behaviour and sexual behaviour to determine potentially altered gestational experiences among strains. Prenatal androgen exposure

has been demonstrated to behaviourally defeminize female SD rats' behaviour (Hoepfner & Ward, 1988). Qualitatively, the SHRs in this study appeared to behave similarly to SD rats in home cages and when handled both prior to and throughout the duration of the experimental protocol. However, the altered sexual reproduction of SHRs has been well documented and has shown a lowered percentage of successful pregnancies, and smaller litters than reference strains (Pinilla et al., 1992). This may be indicative of altered prenatal exposure to androgens. Future studies are required to elucidate whether the observed differences in behavioural sensitisation and reproductive deficiency are caused by altered prenatal androgen exposure.

The present study utilises a behavioural sensitisation protocol as a means of assessing the locomotor response to cocaine. This test is able to measure the innate response of an animal to the sensitising neural and behavioural effects of a psychostimulant and thus is a valuable indicator of the development of addiction over time. The behavioural sensitisation protocol involved repeated exposure to cocaine injections that have been demonstrated to enhance stimulant-induced dopamine release (Hooks et al., 1994). However, this protocol has its limitations in its applicability to humans. The behavioural sensitisation model lacks the ability to determine the extent to which the rodents find cocaine rewarding, which has been assessed using alternative tests such as the aforementioned conditioned place preference test (CPP). Additionally, the cocaine administered in this protocol is delivered by the experimenter and therefore does not replicate the traditional pattern of acquisition of substance use disorders in which the individual is in control of the dose (voluntary intake). To remedy this, an alternative method of administering cocaine, such as self-administration, would allow further conclusions of whether SHRs may find cocaine more pleasurable and thus would be more susceptible to addiction.

It is challenging to draw meaningful conclusions on the expression of cFos with only one brain area and sex having been preliminarily assessed. Future data analysis is necessary to quantify the cells expressing cFos to determine the extent to which the neurological response to cocaine is altered in SHRs. However, the preliminary qualitative findings of the cFos immunohistochemistry in the left nucleus accumbens core of female rats appears to follow a similar pattern of neural response to cocaine as observed in the behavioural sensitisation protocol. This preliminary finding may affirm the altered response to cocaine in SHRs. However, a limitation of the current immunohistochemistry protocol is the lack of specific labelling. cFos is expressed following strong activation of a neuron, and is thus indiscriminate and may include those that are not dopamine-specific. For example, the prefrontal cortex receives input not only from the mesocorticolimbic dopaminergic pathway, but also contains receptors for other neurotransmitters including serotonin, glutamate, acetylcholine, and GABA (Steketee, 2003); thus it can be challenging to determine what specific neurotransmitter is causing the increased cFos staining in this brain region. One method to evaluate whether these stained cells are dopaminergic is to perform additional immunohistochemistry staining for the dopamine D1 and D2 receptors. Both receptor subtypes contribute meaningfully to dopaminergic reward processes (Ikemoto et al., 1997). Therefore future studies involving double staining neurons expressing these receptors and cFos would be required to further elucidate the effects of strong dopamine signalling observed.

In summary, we were able to replicate the hyperactive phenotype in SHRs, though not the inattentive phenotype. We also found that the ambulatory and fine motor responses induced by a behavioural sensitisation to cocaine were enhanced in female SHRs compared to female reference rats which was mirrored in the preliminary qualitative cFos expression of the left

nucleus accumbens core of these rats. Alternatively, behavioural sensitisation to cocaine was diminished in male SHRs compared to male reference animals. This finding further implicates important sex differences in the development of addiction and indicates a need to further explore the increased susceptibility to drug addiction in females with ADHD.

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