The effect of acute stressors on heroin seeking after punishment-imposed abstinence and the role of individual trait variation in male and female rats

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ABSTRACT

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Substance use disorders involve a cyclical pattern of escalating drug use, abstinence from the drug of choice, and subsequent relapse. Relapse can be driven by stressful life events causing drug cravings and preoccupation, leading to the return of escalating drug use. Different models have been proposed to study this phenomenon in animals. Often users abstain due to the negative consequences of drug-seeking and taking. As such, voluntary abstinence models were created whereby animals often choose to abstain of their own volition. One of the ways to induce this is through a punishment-imposed abstinence model, where abstinence is achieved through punishing animals' drug-seeking or drug-taking behaviours. Past research in our laboratory using this model has shown that acute food deprivation stress increases heroin-seeking following abstinence. However, there is little research on the generalization of the effects of acute food deprivation stress on heroin-seeking to other acute stressors using a punishment model. Individual differences in many traits correlate with drug use behaviours. Specifically, traits like reward-seeking, anxiety-like behaviour, and novelty-seeking have been associated with different aspects of drug use. Greater levels of reward-seeking, anxiety-like, and novelty-seeking behaviours are thought to increase the risk of substance use. Finally, sex differences are well documented in addiction research due to biological and sociological reasons. Animal models further support sex differences: females often escalate to drug use faster, have higher intake levels, and relapse more. Furthermore, females are more resistant to punishment than their male counterparts, which may mean that females are more resistant to punishment-imposed

abstinence. The current study had three aims: (1) to investigate the generalization of the effect of acute food deprivation to other acute stressors in a punishment-imposed abstinence model: restraint stress, forced swim stress, and foot-shock stress; (2) to measure the degree to which individual differences predict stress-induced relapse using the following tests: sucrose preference (SP) test, elevated plus maze (EPM), open field test (OF), and tail-flick (TF) test; and (3) assess differences between male and female rats in punishment-imposed abstinence and stress-induced drug-relapse behaviour. Adolescent male and female Long-Evans rats underwent SP, EPM, OF, and TF testing. Subsequently, rats were trained to self-administer heroin (0.1 mg/kg/infusion) under a seek-take chain for at least 14 days. Rats then underwent punishment-imposed abstinence for 8 days. Afterwards, rats were exposed to a stress condition (males: food deprivation, restraint, forced swim, or footshock; females: restraint, forced swim, or food deprivation) before undergoing a heroin-seeking test. It was found that the effect of acute food deprivation stress on relapse generalized only to forced swim-induced relapse in both male and female rats. Individual differences in traits of anxiety, novelty-seeking and reward-seeking did not reliably predict drug relapse. Male and female rats did not differ in punishment-imposed abstinence or stress-induced relapse behaviour.

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Introduction

1.1 Models of Relapse

Drug addiction is a large health concern with significant societal and financial consequences. Per the National Center for Drug Abuse Statistics, the federal budget for drug control was over $35 billion dollars in 2020 alone (Substance abuse and addiction statistics, 2022). In 2020, 91,799 overdose deaths due to illegal drugs use were reported, of which 74.8% were opioid-related deaths (Centers for Disease Control and Prevention, 2022). Overdose deaths have been on the rise. For example, the rate of overdose deaths increased from 2019 to 2020 by 31% (Centers for Disease Control and Prevention, 2022).

Drugs that cause problematic substance use are said to be both rewarding (interpreted to have positive effects) and reinforcing (whereby drug use behaviours are often repeated) (Hyman & Malenka, 2001). Following initial use, drug-use may increase progressively in those vulnerable which in turn causes molecular changes in the brain. These molecular changes further promote continued and increasing drug use. Problematic substance use then follows a chronic course where an individual engages in periods of abstinence and then relapse to active drug use (Hyman & Malenka, 2001). Currently, relapse is the largest challenge facing the treatment of drug use with only a handful of pharmacological treatments approved to prevent it (Daley, 1987; O'Brien & McLellan, 1996).

There are three known triggers to relapse: small "priming" doses of the drug (or drug-re-exposure) (de Wit, 1996), drug-related cues (which could be environments in which drug use is taking place or drug paraphernalia) (Childress et al., 1993), and stress (Sinha, 2001). Studies in both human and animals have shown that craving (Ludwig and Wikler, 1974; Childress et. Al,

1988; Jaffe et al. 1989; Sinha et al., 2000; Preston et al., 2018), and the reinitiation of drug seeking (De Vries et al., 1998; Shalev et al., 2001; Rogers and See, 2007) are induced by these three triggers.

It is important that animal models of drug relapse reflect the human condition to better address rising concerns. Since the 1970s, the most used relapse model has been the reinstatement model. Rodents are trained to self-administer a drug through operant behaviours such as a nose poke or pressing a lever, which is then extinguished in the absence of the drug and cues associated with it. Following extinction, drug seeking (e.g., number of lever presses) is assessed during a reinstatement test following exposure to one of the known relapse-triggers (Shaham et al., 2003). This model has been criticized for not mimicking the way in which humans abstain, as drug seeking in humans does not undergo extinction.

The forced abstinence model imposes abstinence by the experimenter. In this model of relapse, subjects acquire the drug self-administration through similar means (lever or nose-poking reinforced by the drug reward). Subjects are then removed by the experimenter or kept in their operant conditioning chamber without access to the drug (Fredriksson et al., 2021, Reichel & Bevins, 2009). This model appears to have more face validity as it mimics some aspects of drug relapse in humans such as mandated (e.g. hospitalization or incarceration), or inpatient (e.g. rehabilitation clinic) abstinence. As such, a major concern for users who undergo forced abstinence is drug-craving and relapse due to re-exposure to drug related-stimuli or associated contexts (Ehrman et al., 1992; Sinha, 2007).

The reinstatement and forced abstinence models have demonstrated decent postdictive validity (Fredriksson et al., 2021). Medications approved by the Food and Drug Administration (FDA) for nicotine, opioid, and alcohol addiction (e.g., naltrexone, buprenorphine, and

methadone) have subsequently demonstrated decreased reinstatement and relapse in reinstatement and forced abstinence models (Epstein et al., 2006; Sinha, Shaham, & Heilig, 2011; Heilig et al., 2016). However, these models have failed to show sufficient predictive validity (Reiner et al., 2019; Venniro et al., 2020), possibly due to the dissimilarity between the conditions of these models and those surrounding human abstinence (Fredrikkson et al., 2021). Oftentimes, human abstinence is voluntarily self-imposed due to either the negative outweighing the rewarding effects of drug use or the availability of alternative nondrug rewards chosen in place of the drug reward (Marlatt, 1996; Epstein and Preston, 2003). To mimic these conditions more closely, models of voluntary abstinence were developed. There are two major voluntary abstinence paradigms: 1) Abstinence induced by availability of alternative non-drug rewards using discrete choice procedures and 2) Abstinence induced by negative consequences of drug taking and seeking.

In alternative choice models, palatable food or social interaction are often used to induce abstinence (Venniro et al., 2018; Venniro and Shaham, 2020). Voluntary abstinence occurs by introducing a mutually exclusive choice between the nondrug and drug reward. During the abstinence relapse test subjects are tested for relapse to drug-seeking in the absence of the alternative (Venniro and Shaham, 2020). In human drug users, the rewards competing with drug use are most often social reward (such as seeing family, friends or maintaining employment) (Venniro et al., 2018). Criticisms of these models are that negative consequences, a leading cause of abstinence in humans, are not represented.

In abstinence models involving adverse consequences there are two main procedures: 1) Electrical barrier-induced voluntary abstinence and 2) Punishment-induced voluntary abstinence. For electric barrier procedures there are three main phases: drug self-administration, electric

barrier-induced voluntary abstinence, and relapse tests. Initially, subjects are trained to self-administer a drug. During voluntary abstinence, drug-seeking is suppressed with the use of an electric barrier of increasing intensity in front of the drug-paired apparatus. To perform operant behaviour paired with drug self-administration, subjects must cross the barrier. Relapse tests are often, but not always, administered in the absence of the electric barrier (Cooper et al., 2007; Peck et al. 2013). This model associates the negative consequences with drug-seeking as the electric barrier is encountered prior to the drug-paired lever and mimics abstinence in humans due to the adverse consequences of drug seeking (e.g., financial obstacles to obtaining the drugs, stressful interactions with law enforcement, family, or drug dealer, etc.) (Cooper et al., 2007). A criticism of this model is that drug seeking efforts are always associated with negative events, which is unlike the human condition. Another is that drug taking may overlap with the electric footshock as the subject crosses over to the safe zone following the infusion resulting in the punishment of the taking response.

Punishment-induced voluntary abstinence occurs with the suppression of drug-taking behaviour by response-contingent footshock, usually, but not always, in the self-administration context (Marchant, Li, & Shaham 2013). Oftentimes punishment occurs on a certain percentage of trials in the place of the administration of the drug or concomitantly with drug infusion, and non-punished drug taking occurs in the remainder of the time. Relapse tests are conducted in the absence of shock. In this model, negative consequences are associated with the operant response assigned to drug taking (Krasnova et al., 2014; Marchant, Li, & Shaham 2013). Thus, the model mimics abstinence in humans due to the consequences of taking rather than seeking (i.e. loss of employment, living quarters or social bonds). This model has received criticisms because in human behaviours associated with drug seeking are dissociated from those leading to drug taking

(Peck & Ranaldi, 2014). In fact, drug seeking is usually the risky behaviour (such as financial burdens, run-ins with law enforcement, dangerous circumstances in seeking out the drug, etc.) (Peck & Ranaldi, 2014). Additionally, having a punishment contingency that immediately follows the administration of a drug reduces the effectiveness of the shock as a punisher (Panlilio, Thorndike, & Schindler, 2005; Pelloux, Everitt & Dickinson, 2007). In order to address these criticisms, a variation of the model was developed that introduces a seek-take chain where seeking and taking responses are dissociable. In this way, only the seeking response is punished, and only on a percentage of trials (Pelloux, Everitt & Dickinson, 2007, Chen et al., 2013, Krasanova et al., 2014). On trials that are punished, drug taking responses are usually not available, and the drug is not administered (Pelloux, Everitt & Dickinson, 2007). Currently, the punishment-imposed abstinence model has been validated with cocaine (Pelloux et al., 2018; Farrell et al., 2019), methamphetamine (Krasnova et al., 2014; Torres et al., 2017), alcohol (Marchant, Li, & Shaham 2013; Marchant et al., 2014; Campbell et al., 2019), and opiates (Panlilio, Thorndike, & Schindler, 2003; Panlilio, Thorndike, & Schindler, 2005), but not specifically with heroin. Due to the greater translatability of the model, we decided to use punishment-imposed abstinence to assess heroin-seeking.

1.2 Stress and Relapse

Drug craving or "wanting" is a key feature in the cycle of addiction and is involved in maintaining continued drug use (Sinha, 2008). Neuroadaptations resulting from chronic drug use are thought to underlie craving (Robinson & Berridge, 1993). These neuroadaptations subsequently increase the incentive salience of drugs and their associated cues, leading to increased craving or "wanting" in their presence (Robinson & Berridge, 1993). In humans, studies show that stressful life events can lead to drug cravings and relapse (Shiffman & Wills,

1985; Kreek & Koob, 1998; Sinha, Catapano & O'Malley, 1999; Khantzian, 1985; Kosten et al., 1986). Stress is the processes related to perception, appraisal, response, and adaptation to threatening events and stimuli (Sinha, 2008). Stress and withdrawal states related to drug use have been shown to elicit craving, relapse, anxiety, and negative affect, and to increase drug-cue reactivity (Childress et al., 1994; Cooney et al., 1997; Sinha, Catapano, & O'Malley, 1999; Sinha et al., 2000; Fox, Bergquist, Hong, & Sinha, 2007; Hyman et al., 2007; Sinha, 2007).

There are two types of stress states: chronic and acute. Chronic stress occurs over long periods and can be conceptualized as spending time long term in toxic environments, fighting with a spouse constantly, a divorce process, etc. Acute stress is short-term and is usually a singular event. Examples of this type of stress are a home break-in, a traffic jam, receiving criticism at work, a singular fight with a loved one, etc. Findings indicate that stressful events accumulated over a lifetime significantly predict dose-dependent drug and alcohol use (Turner & Lloyd, 2003; Lloyd & Turner, 2008). In animal studies, exposure to acute or chronic stress may enhance the reinforcing efficacy of drugs, and increase self-administration and reinstatement of extinguished drug seeking (Shaham & Stewart, 1994; Miczek, Mutschler, & Mizcek, 1996; Lu, Shepard, Hall, & Shaham, 2003; Garcia-Keller et al., 2016; Mantsch et al., 2016).

Acute food deprivation stress is a particularly interesting stressor due to its temporal properties. Usually, acute stressors occur over the course of a few hours or less (restraint stress, forced swim, footshock, social defeat, etc.). Acute food deprivation stress, however, occurs over 24 hours and thus may be between an acute and chronic stressor. In the reinstatement model, Shalev and colleagues have found acute food deprivation stress reinstates both heroin (2000) and cocaine (2003) seeking in male rats. This effect has further been found with morphine conditioned-place preference (CPP) (Mozafari et al., 2020). Our laboratory recently

demonstrated food deprivation-induced heroin relapse following punishment-imposed abstinence (Borges, Charles & Shalev, 2022). Due to the uniqueness in temporal properties of acute food deprivation, we wondered if its effect on heroin-seeking under a punishment-imposed abstinence paradigm would generalize to other, shorter acute stressors such as footshock, forced swim, and restraint.

A common method of administering acute stress in rodents is acute footshock stress. In operant conditioning chambers or other housing chambers, grid floors are used where inescapable footshock can be administered, with intensities ranging from mild to moderate. Using the reinstatement model, intermittent footshock was found to reinstate drug-seeking in heroin (Shaham & Stewart, 1995), cocaine (Erb et al., 1996), alcohol (Le et al., 1998), nicotine (Buczek, Le, Wang, Stewart, & Shaham, 1999), and methamphetamine (Shepard et al., 2004) seeking.

Forced swim stress is an acute stressor where a rodent is dropped into a clear cylindrical container where they are forced to swim to stay afloat, with no method of escape. Conrad et al. (2010) showed that 4-5 min cold swim stress reinstated extinguished cocaine seeking for up to three days following stress exposure. In a study by Farzinpour, Taslimi, Azizbeigi, Karimi-Haghighi, and Haghparast (2019), forced swim stress induced the reinstatement of extinguished morphine-conditioned place preference in male rats. Anderson, Lopez, and Becker (2016) found that in a self-administration model, ethanol intake escalated in dependent but not nondependent mice following forced swim stress using a chronic intermittent ethanol drinking paradigm. Jackson, McLaughlin, Carroll, and Damaj (2013) showed that forced swim stress significantly increased reinstatement of nicotine-conditioned place preference.

Restraint stress is conducted using a transparent plexiglass tube where a rodent's movement is severely restricted. This method has shown mixed results in the literature regarding its effectiveness. Shalev and colleagues (2000) showed that restraint stress of 5, 15, or 30 minutes administered outside the self-administration context did not significantly alter the reinstatement of heroin seeking. In another study by De Giovanni et al. (2016), 30 and 60, but not 15-min restraint stress reinstated cocaine-conditioned place preference in rats, while Ribeiro Do Couto and colleagues (2006) found the same effect with morphine CPP. In another study by Taslimi, Komaki, Sarihi, and Haghparast (2019), acute (3 hr) and chronic (1 hour for seven days) restraint stress reinstated methamphetamine-induced CPP in male Wistar rats. Restraint stress also reinstated extinguished nicotine CPP (Leão, Cruz, & Planeta, 2009). The lack of effect demonstrated by Shalev et al. (2000) may have resulted from either the restraint context (i.e. out of drug-taking context) or due to the difference in the procedure (conditioned place preference vs. intravenous self-administration).

We hypothesized that acute food deprivation would generalize to acute footshock, restraint, and forced swim stress and increase heroin-seeking following the punishment-imposed abstinence procedure.

1.3 Personality characteristics and Individual Differences in drug seeking and relapse

Individual differences in the vulnerability to develop substance use disorder are thought to be present before one engages in drug use and may be related to one's sensitivity and proclivity to drug reward (Haertzen et al., 1983). These differences may be due to genetic or environmental differences or a combination of the two. Heritability studies have established the important role of genetics (Agrawal & Lynskey, 2008). A study by Bierut and colleagues (1998)

found that siblings of those with alcohol, cannabis, cocaine, and nicotine had higher rates of dependence in those same drug classes than controls. Individuals more susceptible to SUD may differ in personality or individual factors, and there is evidence that social influence moderates this interaction (Bardo, Neisewander, & Kelly, 2013). Although a single predisposing personality type to substance use disorder is unlikely, there has been research into finding potential personality risk factors for developing substance use disorders (Cox, 1987).

There have been many studies that have shown a relationship between trait anxiety, anxiety sensitivity and the misuse of substances (Hearon et al., 2011; Lejuez et al., 2006; McHugh et al., 2017; Rogers et al., 2019; Schmidt, Buckner, & Keough, 2007; Stewart et al., 1997). Pathological anxiety occurs when non-threatening situations cause individuals a long-lasting maladaptive anxiety response (Jupp, Jones, & Dalley, 2019). Anxiety disorders and problem substance use can present comorbidly (Grant et al., 2004; Swendsen et al., 1998). This relationship is thought to be bidirectional, whereby anxiety disorders can increase the risk of substance use disorders, and substance use disorders can increase the risk for anxiety disorders (Kushner, Abrams, & Borchardt, 2000). In terms of relapse, Schellekens, de Jong, Buitelaar, and Verkes (2015) found that male alcohol-dependent individuals with comorbid anxiety disorders were prone to early relapse during the first three months of treatment. In rodent studies, findings have been more mixed. Hayton, Mahoney, and Olmstead (2012) found that in adult male Long-Evans rats, time spent on the open arms of the EPM (an anxiety indicator) predicted alcohol consumption during limited (1 hour/day) access to alcohol as well as the escalation of intake during 72 hour continuous access. A similar finding by Walker et al. (2009) showed adult male and female Sprague Dawley rats who spent less time in the open arm of the elevated plus-maze consumed higher doses of cocaine/saccharin solution, accounting for 25% of the variance in

cocaine consumption. This association was not found for natural reward (saccharin only) consumption. Abreu-Villaça and colleagues (2006) used male and female adolescent C57BL/6 mice. They found anxiety-like behaviour (as tested by the percentage of center squares crossed in the hole board activity box) was not a good predictor of nicotine consumption. These differences may be due to different drug classes, behavioural procedures, age, or species.

Novelty-seeking or sensation-seeking is argued to predict drug use in humans (Kosten et al., 1994; Zuckerman, 1994; Wills, Windle & Cleary, 1998; Ball, 2004). Zuckerman (1994) defines novelty/sensation-seeking as the willingness and drive to seek out variable, novel, and intense experiences despite the negative social, legal, or financial risks. It is thought that drug use increases novelty seeking (Ersche et al., 2010). Horvath and group (2004) found that using a longitudinal design, higher levels of sensation-seeking in adolescents (14-15 years old) are associated with higher levels of substance use in young adults (19-21 years old). Additionally, effects were bidirectional, with earlier substance use linked with higher levels of sensation seeking in young adulthood (Horvath et al., 2004). In preclinical studies, Walker and colleagues (2009) assessed novelty-seeking in male and female Sprague-Dawley rats of various ages by measuring locomotor activity in a novel open-field arena. They found that higher activity levels in the novel environment were significantly correlated with higher cocaine/saccharin solution consumption in adolescents, accounting for 18% of the variance. This relationship was not found in adults (Walker et al., 2009), indicating higher novelty-seeking predicts higher levels of substance use in adolescents but not adult rats. In another study by Belin and colleagues (2011), high novelty preference (HNP) rats, as identified through the propensity to choose a new environment in a free-choice procedure, were found to have higher cocaine addiction scores. Abreu-Villaça et al. (2006) found that high-novelty seeking adolescent mice (postnatal day 30)

had increased consumption of nicotine solution as compared to low novelty seekers. There are mixed findings as to whether alcohol use is related to novelty-seeking. Manzo and colleagues (2014) found that Roman rats bred for high (RHA-I) or low (RLA-I) active avoidance learning differed in their novelty-seeking and ethanol consumption. RHA-I rats showed a higher preference for ethanol over water and higher novelty-seeking measures in the hole-board and Y-maze tests. In contrast, Bienkowski, Koros, and Kostowski (2001) did not find a correlation between novelty-seeking measures in the open-field or novel object test and oral ethanol self-administration in adult male Wistar rats.

Another dimension that may correlate with the propensity to engage in problematic substance use is the proclivity for natural rewards such as sweet taste preference (Carroll et al., 2008, Dess et al., 1998). In humans, Kampov-Polevoy, Garbutt, and Janowsky (1997) found that 65% of men with alcohol use disorder preferred solutions with the highest sucrose concentrations compared with 16% of the nonalcoholic group. Janowsky, Pucilowski, and Buyinza (2003) found that cocaine-dependent patients preferred the highest concentration of sucrose solution at a higher percentage than their depressed non-using counterparts (who preferred the lowest concentration of sweet solution). In a study by Garfield and Lubman (2021), they suggest that reduced sensitivity to low sweetness levels and an increased sweet preference may correlate with more severe opioid dependence scores. Furthermore, in those who had maintained prolonged abstinence to opioids there was a shift towards preference of sweeter flavours. In preclinical studies, Sprague-Dawley rats that were selectively bred or selected for high sweet-intake (HiS) consumed more cocaine, ethanol, and morphine solution than rats that were bred or selected for low sweet intake (Los) (Carroll et al., 2008). Furthermore, the HiS rats acquired drug self-administration faster, self-administered more drugs during short (2 hr) access, escalated their

drug-intake quicker during long (6 hr) access, showed greater resistance to extinction, and showed higher levels of cocaine-priming induced reinstatement (Carroll et al., 2008).

Given that trait-anxiety/anxiety-sensitivity, novelty-seeking, and preference for sweets have an established relationship with substance-use disorders and addiction-like behaviours, we wondered whether individual variation regarding these dimensions would predict the degree to which rats relapse following acute stress using a punishment-imposed abstinence paradigm. We hypothesize that rats with higher anxiety-like behaviour, higher novelty preference, and higher sweet preference will have increased acute-stress-induced relapse to heroin-seeking following punishment-imposed abstinence. In addition to the above personality characteristics, we measured pain sensitivity to elucidate the connection between punishment learning and pain sensitivity. We wondered how it might predict relapse behaviour. Rats with higher pain sensitivity may be more resistant to footshock punishment and, therefore, more susceptible to relapse due to less negative associations of pain with heroin seeking.

1.4 Sex differences in animal models

Males and females differ in biologically and sociologically measurable ways. Sex differences present at each stage of the addiction cycle and are often quantitative. Quantitative differences are when males and females differ on the magnitude of their response, wherein the trait is the same but one sex may have a more significant response.

In the binge/intoxication phase men use more of drugs, regardless of drug category (Substance Abuse and Mental Health Services Administration, 2007) but women are thought to telescope and escalate their drug use faster than men (Kosten et al., 1994; Brady and Randall, 1999; Westermeyer & Boedicker, 2000; Becker and Hu, 2008; Cooper and Haney, 2014). In preclinical research, female rodents escalate drug use quicker and take more drug than male

rodents (Lynch and Carroll, 1999; Cicero et al., 2002; Karami and Zarrindast, 2008). Lynch and Carroll (1999) found that female Wistar rats acquired self-administration more rapidly and self-administered more heroin and cocaine than males.

In both clinical and preclinical studies, sex differences in opiate relapse show mixed results. Ignjatova and Raleva (2009) found women relapsed more than men, whereas Gordon et al. (2017) found lower relapse rates in women following 1 year of buprenorphine treatment. In preclinical work, Malone and colleagues' (2021) trained male and female Sprague-Dawley rats to self-administer fentanyl across short- (1 hr; ShA) and long- (6 hr; LgA) access sessions. They found higher fentanyl-induced reinstatement in females after ShA but not LgA, lower cue-induced reinstatement in females after LgA but not ShA, and no sex differences following yohimbine-induced reinstatement. Fulenwider et al. (2020) used a short-access oxycodone self-administration paradigm and found no sex differences in footshock-induced reinstatement.

In addition to sex differences in the stages of addiction, sex differences exist in other behavioural aspects like reward-sensitivity and susceptibility to punishment. Studies have shown that women are better at delaying reward gratification than men (Byrnes, Miller, & Schafer, 1999; Silverman, 2003). A preclinical study by Chowdhury et al. (2019) demonstrated no sex differences in reward-related associative learning but did show that females were faster in punishment-avoidance learning. Afterwards, females were more sensitive to probabilistic punishment and less sensitive compared to males when punishment could be avoided. Sutton et al. (2021) reported that female rats suppressed punished alcohol-seeking when it was combined with an alternative reward. However, when challenged with only punishment, the females showed less suppression of alcohol seeking. This difference did not occur in males. This suggests that females are less sensitive to punishment, under certain conditions.

Given the differences between males and females in all addiction stages and in punishment and reward, our group had the following questions: (1) Will females be more resistant to punishment than males throughout punishment-imposed abstinence; (2) Will females increase heroin-seeking following food deprivation-stress under a punishment-imposed abstinence paradigm, similar to male rats?; and (3) Will other acute stressors (restraint and forced swim) increase heroin-seeking in females, similarly to males? We hypothesize that female Long-Evans rats will show increased resistance to punishment during the punishment-imposed abstinence and show a greater propensity to relapse to heroin-seeking following acute stress.

Methodology

1. General Protocol

1.1. Subjects

Adolescent male (250-275g; N=60) and female (220-240g; N=20) Long Evans rats (Charles River, St. Constant, Quebec, Canada) were initially double housed in the animal care facility (ACF) at Concordia University, Montreal, Canada, until two days before the initiation of the pre-screening tests. Rats were maintained under a reverse light cycle (lights off: 9:30 am, lights on: 9:30 pm). Subjects were handled daily for the entirety of the experiment around 9:00 am.

1.2. Intravenous surgery

Before surgery, rats were weighed to ensure they were at the required surgery weight (300 g for males and 230 g for females). They were administered 2 mL of 0.9% saline, penicillin (450 000 IU/rat, s.c.), and atropine (0.1 mg/kg/rat) to aid in hydration and prevent infection. Under 2% isoflurane anesthesia, three and a half centimeters of silastic catheter (Dow Corning, Midland, MI, USA) was inserted through a small incision in the right jugular vein, which was then secured with silk sutures. The remainder of the catheter was then inserted and pulled through subcutaneously to the skull where it was attached to a modified 22-gauge cannula (Plastics One, Roanoke, VA). It was then anchored to the skull using dental cement secured to 5 jeweler's screws. Rats were administered the analgesic ketoprofen (5.0 mg/kg; Merial Canada) postoperatively, and for 2 days following surgery. Catheters were flushed daily with heparin/gentamicin (7.5 IU + 0.8 mg/rat) in sterile saline to prevent blockage and infection.

1.3. Apparatus

Behavioural experiments were performed in standard operant conditioning chambers with two retractable levers, a house light, white cue lights above the levers, and a tone generator (2.9 kHz; Coulbourn Instruments, Allentown, PA, USA; 29.0 cm × 29.0 cm × 25.5 cm). One lever was named take lever and was paired with a heroin infusion. The other lever was named seek lever, and it allowed access to the take lever. The infusion pump was connected to the catheter through a liquid swivel (Lomir Biomedical) and Tygon tubing (Saint-Gobain) shielded with a metal spring.

1.4.1 Pre Screenings

1.4.1.1. Sucrose Preference

The sucrose preference test was performed daily at 9:00 am during their dark cycle. Rats were single housed in the ACF. On day 1, two bottles of tap water were weighed and placed on opposite sides of an animal's cage. On day 2, each bottle of water was weighed and recorded. The side preference of the rodent was determined by the percent of water consumed from the left versus the right side. One bottle of water was then switched out with a bottle of 1% sucrose solution. The water and sucrose bottle were weighed before being placed on opposite sides of the cage. The sides each bottle was kept on were counterbalanced among the rats. On day 3, the bottle weights were recorded, and the sides of the water and sucrose were counterbalanced. On day 4, the bottle weights were recorded, and the bottle of sucrose solution was removed from the cages. Percent sucrose preference was determined by the percent sucrose-solution consumed compared to the percent water consumed.

1.4.1.2. Elevated Plus Maze (EPM)

The elevated plus-maze used in experiments was made of plywood and consisted of two open arms and two closed arms (50.0 cm long x 10.0 cm wide, with 40.0 cm high walls) that extended from a central platform which was 50.0 cm above the floor.

Rats were tested during their dark phase and placed at the junction of the open and closed arms facing the open arm opposite to the experimenter. Rats' behaviour was recorded and analyzed using the ANY-maze video tracking software for 10 minutes. The apparatus was cleaned with 70% ethanol between tests. The number of entries into and the time spent in the open and closed arms were recorded, as well as time spent rearing, grooming, freezing, time the head was in the center zone (termed a head-dip), and distance travelled in meters. The percentage of time spent in the open arms was interpreted as a measure of fear and anxiety, where the more time spent in the open arms, the less anxious the animal was (Cruz, Frei, & Graeff, 1994). Time spent rearing (Lever, Burton, O'Keefe, 2006) and distance travelled was used as a measure of novelty seeking. Time spent freezing was a measure of fear and anxiety (Hart et al., 2010).

1.4.1.3. Open Field (OF)

The circular open field arena used in experiments was made of dark grey plastic and had a diameter of 1.0 m and a height of 0.5 m. The bottom consisted of Sani-chip bedding.

Rats were tested during their dark phase and placed near the edge of the open field arena facing the same direction. Rats were recorded for 30 minutes using the ANY-maze video tracking software. Behaviours recorded were distance travelled, time spent in the outer edge, time spent in the middle zone (diameter of 0.5 m), and time spent freezing and rearing in both outer and middle zones. Time spent in the center, time spent rearing, and distance travelled were thought to measure novelty-seeking. Time in the center was also a measure of anxiety, as rodents prefer enclosed and not open spaces.

1.4.1.4. Hot Immersion Test Protocol

A beaker with 800 mL of tap water was heated over a hot plate using a magnetic stirrer until it reached between 52 and 55ºC. Rats were wrapped in a dry towel with only their tail exposed. To measure pain sensitivity, rats' tails were submerged 2.0 cm in the water and left in the water for the duration of the time it took for the rat's tail to flick, or a maximum of 20 seconds. This was recorded using a video recorder and a timer.

1.4.2. Self-administration

1.4.2.1. Self-administration with taking lever under fixed ratio 1 (FR1)

Each trial began with the insertion of the take lever. Once the rat pressed the take lever once (FR1), the take lever retracts, the cue light above the lever and tone turned on for 20 s, the houselight was turned off, and a heroin infusion (0.1 mg/kg) was delivered. A 30 s inter-trial interval (ITI) followed in which no cues (cue light and tone) were presented, the houselight was off, and the lever was still retracted. For the beginning of the next trial, the take lever was inserted again while the houselight was turned on, indicating the availability of the drug. The daily sessions lasted six hours, and training was performed for three days.

1.4.2.2. Self-administration with a seek-take chain under FR1

In this phase, the seek lever was introduced. The trial started with the insertion of only the seek lever (with the take lever retracted) while the houselight was on. Once the rat pressed one time (FR1) on the seek lever, this lever was retracted, and the take lever was inserted right away. When the rat pressed one time on the take lever, the take lever was retracted while the light cue and tone were on for the 20 s, the houselight turned off, and a heroin infusion (0.1 mg/kg) was delivered. Then, a 30 s ITI followed as in the previous phase. The next trial began with the

insertion of the seek lever and illumination of the houselight. If a rat failed to complete the seek-take chain for 10 min (did not press the seek lever or the take lever), the 30 s ITI followed to represent the end of a trial and the loss of the opportunity to administer the drug. After the 30 s ITI, the seek lever was extended again to begin another trial. The daily training sessions lasted six hours, performed for three days.

1.4.3. Self-administration with a seek-take chain under Variable Intervals (VI5, VI30 and VI60)

This phase was similar to the one explained above (see Self-administration with a seek-take chain under Fixed Ratio 1 (FR1)), but a variable interval 5 (VI5) schedule was introduced on the seek lever instead of FR1.

For the VI5 schedule, the software randomly selected a time from a list of 0.1 s, 5 s and 10 s, which resulted in an average of 5 s. The first press on the seek lever began the VI5 schedule, and the first press after the variable interval led to the extension of the take lever. Once the take lever was inserted and the rat pressed one time, the take lever was retracted while the light cue and tone were on for 20 s, the houselight was off, and a heroin infusion (0.1 mg/kg) was delivered. Then, a 30 s ITI followed as in the previous phase. The next trial began with the insertion of the seek lever and illumination by the houselight. If a rat failed to complete the seek-take chain for 10 min (not pressing the seek lever or the take lever), the 30 s ITI followed to represent the end of a trial and the loss of the opportunity to administer the drug. After the 30 s timeout, the seek lever was extended again to begin another trial. VI5 training lasted for two days. Next, rats were trained under a similar schedule but with VI30 for the seeking link for four days and another four days with the VI60 schedule. For the VI30 schedule, the software randomly selected a time from

a list of 15 s, 30 s and 45 s, for an average of 30 s. For the VI60 schedule, the software randomly selected a time from a list of 45 s, 60 s and 75 s.

The ITI varied along with the different schedules. It began at 30 s for FR1 and VI5 but gradually increased to 3 min during VI30 and VI60, the last training session performed at VI60 schedule with an ITI of 5 mins. Self-administration training continued for approximately 22 days.

1.4.4. Punishment-Imposed Abstinence

After reaching the self-administration criterion (constant number of infusions over two training days under VI60), mild footshocks were introduced to punish drug-seeking on 30% of completed seek links. The trial began with the seek lever inserted and the house light on. The seek lever was under a VI60 as described above. After the seek link was completed, the lever was retracted, and the software selected to administer either a mild footshock on 30% of the trials or extended the take lever on 70% of the trials. The footshock was followed by a 5 min ITI (the houselight and all cues were off). Responses on the take lever (on trial when it is available) resulted in a heroin infusion (0.1 mg/kg), the presentation of the cues – white light and tone – for 20 s, turning the houselight off, and a 5 min ITI. The next trial began with the insertion of the seek lever and the illumination of the houselight. The daily sessions lasted 6 hours, and it was performed for eight training days.

The footshocks gradually increased from 0.2 mA to 0.6 mA and remained at 0.6 mA until the end of punishment-imposed abstinence. The criterion for abstinence were less than five infusions over two consecutive days.

1.4.5. Heroin-seeking test

On the test days, following exposure to one of the stressors' conditions (see below), rats were connected to the infusion line and underwent a 1-hour session under a VI60 schedule ITI 5 min with no footshock and no heroin infusion.

2. Experiment 1: Generalization of food deprivation-induced relapse to other acute stressors in male rats

2.1. Stressors

2.1.1. Experiment 1a. Restraint Stress

Following the punishment-imposed abstinence phase, rats were left in self-administration boxes with no protocol running for 72 hours with unlimited access to water and food.

Rats were then exposed to the two experimental conditions, in counterbalanced order, 48 hours apart: (1) Restraint stress: rats were placed in a restraint tube (30.0 cm in length, 6.0 cm in diameter) in their self-administration boxes for 30 min prior to a heroin-seeking test. (2) Control: rats were left undisturbed in self-administration boxes for 30 min before the heroin-seeking test.

2.1.2. Experiment 1b. Forced Swim Stress

Following the punishment-imposed abstinence phase, rats were left in self-administration boxes with no protocol running for 72 hours with unlimited access to water and food.

Rats were then exposed to the two experimental conditions, in counterbalanced order, 48 hours apart: (1) Forced swim stress: rats were placed in a water tank (45.0 cm height, 25.0 cm diameter) filled three-quarters of the way with water between 20 and 25 °C for 20 min prior to a heroin-seeking test. They were monitored through a video camera during this time. Afterwards, rats were dried gently with a towel and placed back in self-administration boxes; (2) Control: rats were moved from self-administration boxes to a holding chamber for 20 min prior to heroin

seeking test to control for a contextual change. Afterwards, they were put in self-administration boxes.

2.1.3. Experiment 1c. Intermittent Foot-shock Stress

Following the punishment-imposed abstinence phase, rats were left in self-administration boxes with no protocol running for 72 hours with unlimited access to water and food.

Rats were then exposed to the two experimental conditions, in counterbalanced order, 48 hours apart: (1) Intermittent footshock stress: rats remained in the self-administration box and exposed to intermittent foot shock at an individually determined intensity for 10 minutes on a VI 40 s (where the shock was on for 0.5 s and off for a range of 10-70 s, averaging 40 s); (2) Control: rats were left in self-administration boxes for 10 min prior to heroin-self administration testing with no intermittent footshock.

2.1.4. Food deprivation tests

After the second heroin-seeking test following acute stress, rats were exposed to the two following experimental conditions, in counterbalanced order, 48 hours apart, and heroin seeking was tested again: (1) Food deprived: rats had no access to food for 24 hours; (2) Sated: rats had unlimited access to food for 24 hours. Thus, heroin seeking was tested four times, with food deprivation-induced relapse (or sated condition) always as the second pair of tests.

3. Experiment 2: Generalization of food deprivation-induced relapse to other acute stressors in female rats

3.1. Estrus Cycle Testing

Throughout experiment 2, the estrus cycle was monitored, whereby between 8:30 and 9:00 am daily, during the dark cycle, vaginal secretion was collected with a plastic pipette filled with 20

µl of distilled water by inserting the tip superficially into the rat's vagina and pipetting the liquid 3-4 times. Vaginal fluid was placed on glass slides. Unstained material was observed under a light microscope, without the use of condenser lenses, with 10 and 40X objective lenses. Three types of cells could be viewed: (1) round and nucleated epithelial cells; (2) irregularly shaped cornified cells lacking a nucleus; and (3) small round leukocytes. The proportion of the three types of cells was used to determine the estrous cycle phases (Marcondes, Bianchi, & Tanno, 2002). A sample with mostly epithelial cells was determined to be proestrus. Samples with mostly cornified cells were determined to be in estrus. Samples with an equal distribution of all three cells were determined to be in metestrus. Samples with more leukocytes than the other three cells were determined to be in diestrus.

3.2. Acute Stress

After female Long-Evans rats progressed through all of the general protocols, they underwent acute food deprivation and one of two acute stressors (restraint or forced swim).

3.2.1 Acute Food Deprivation

Following the 72 hour period after punishment-imposed abstinence, rats were exposed to the two following experimental conditions, in counterbalanced order, 48 hours apart, and heroin seeking was tested after each condition: (1) Food deprived: rats had no access to food for 24 hours; (2) Sated: rats had unlimited access to food for 24 hours.

3.2.2. Experiment 2a. Restraint Stress

Seventy-two hours after female rats underwent the last heroin-seeking test following the food deprivation protocol, rats were exposed to the two experimental conditions, in counterbalanced order, 48 hours apart: (1) Restraint stress: rats were placed in a height restraint tube in their self-

administration boxes for 60 min prior to a heroin seeking test; (2) Control: rats were left in self-administration boxes for 60 min prior to the heroin-seeking test.

3.2.3. Experiment 2b. Forced Swim Stress

Twenty-four hours after female rats underwent the last heroin-seeking test following the food deprivation protocol, rats were exposed to the two experimental conditions, in counterbalanced order, 48 hours apart: (1) Forced swim stress: rats were placed in tank filled three-quarters of the way with water between 20 and 25 °C for 4 min prior to a heroin-seeking test. They were observed from a video camera in the other room during this time. Afterwards, rats were dried gently with a towel and placed back in self-administration boxes; (2) Control: rats were moved from self-administration boxes to a holding chamber for 4 min prior to heroin seeking test to control for a contextual change. Afterwards, they were put in self-administration boxes, and a heroin-seeking test was performed.

4. Disposition

Immediately following the last heroin-seeking test, the animals were humanely euthanized. Physical euthanasia was performed following the use of the CO2 system in the Animal Care Facility.

5. Statistical Analyses

All analyses were conducted using GraphPad Prism 9.1.0.

Changes in heroin seeking and taking between the average of the VI60 days of self-administration (baseline) as well as all 8 days of punishment were analyzed separately for male and female rats with a one-way repeated measures ANOVA with the within subjects factor of *punishment day*.

Relapse tests: For both experiments, two-way ANOVAs were used to compare heroin seeking (number of responses on the seek lever) following exposure to the different stressor types with a within subject factors of *stressor type* (food deprivation vs. restraint or forced swim or footshock) and *treatment conditions* (stress, control). The threshold for statistically significant results was set at $p < .05$. Effect sizes were calculated using partial eta square and Cohen's *d*. Mean differences (MD) between conditions and standard errors (SE) were also reported when comparing groups.

In males and females, we performed simple linear regressions to evaluate whether pre-screening measures predicted relapse behaviour following forced swim stress and acute food deprivation stress (for the group of rats who performed food deprivation paired with forced swim). Only stressors that effectively induced relapse were included in these analyses. The pre-screening measures analyzed were as follows: percent sucrose preference, latency to tail-flick, time spent in the open arms of the EPM (time spent in the closed arms is a reciprocal of this measure and thus was not reported), time spent freezing in the EPM, time spent grooming in the EPM, distance travelled in the EPM, distance travelled in the OF, time spent in the center of the OF, time spent freezing in the OF, and fecal boli deposited in the OF.

Results

Experiment 1. Generalization of food deprivation-induced relapse to other acute stressors in male rats

Self-Administration training: All rats (N=52) increased seek lever presses as the variable interval increased across training days. Rats administered a consistent and reliable number of heroin infusions over the training days (Fig. 1).

Punishment phase: During punishment, rats decreased the number of seek lever presses and infusions as the footshock intensity increased over the 8 days (Fig. 2). Using a one-way repeated measures ANOVA, comparisons between the number of infusions and seek lever responses made throughout punishment days and baseline scores during self-administration (average of seeking and taking responses on days under VI60) showed a significant change in the number of seek lever presses and infusions ($F(2.639, 139.9) = 25.26$, $p < .0001$, $\eta_p^2 = 0.323$, $F(3.937, 204.7) = 56.21$, $p < .0001$, $\eta_p^2 = 0.52$, respectively). A post hoc Dunnett's multiple comparisons test was run against the baseline self-administration and each of the 8 days of shock. Seeking responses baseline was significantly higher from seek scores of day 4 (shock 0.5 mA), day 5 (shock 0.6 mA), day 6 (shock 0.6 mA), day 7 (shock 0.6 mA), and day 8 (shock 0.6 mA) of punishment-imposed abstinence (all p's < .002; Fig. 3). Infusions baseline was also significantly higher from infusions on each of the 8 days of punishment-imposed abstinence (all p's < 0.003; Fig. 3).

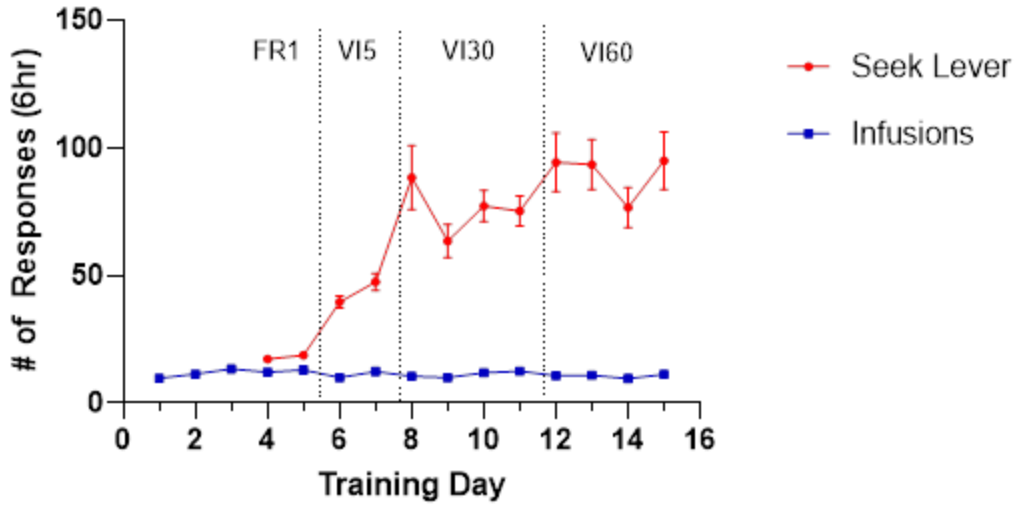

Figure 1. Mean (±SEM) number of seek lever presses (in red) and heroin infusions (in blue) throughout the duration of self-administration training separated by reinforcement schedule (FR1, VI5, VI30, VI60) in male rats (n= 52).

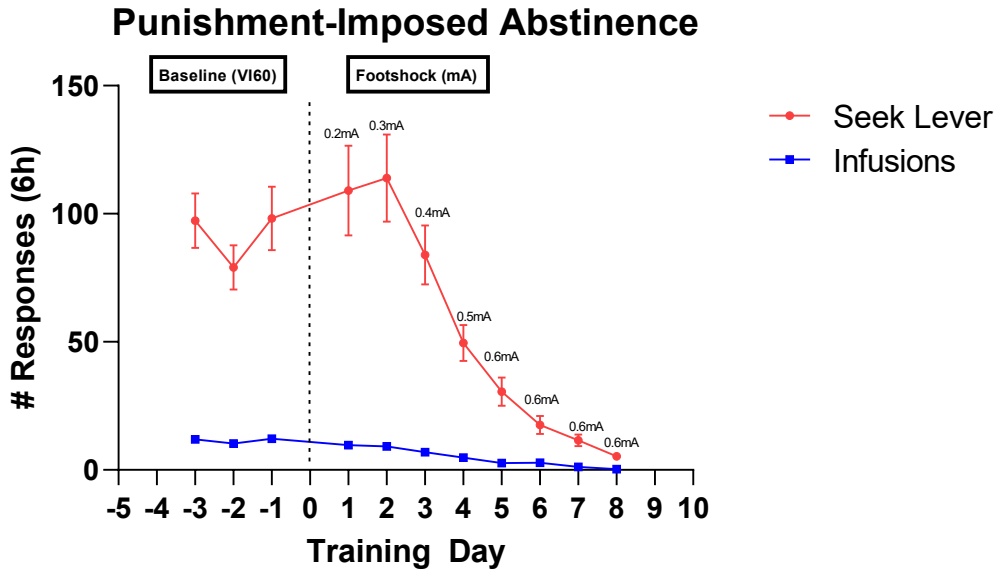

Figure 2. Mean (±SEM) number of seek lever presses and infusions for males on the last three days on VI60 schedule (Baseline) followed by punishment-imposed abstinence (Footshock). The footshock intensity increased by 0.1mA per training day (n= 52)

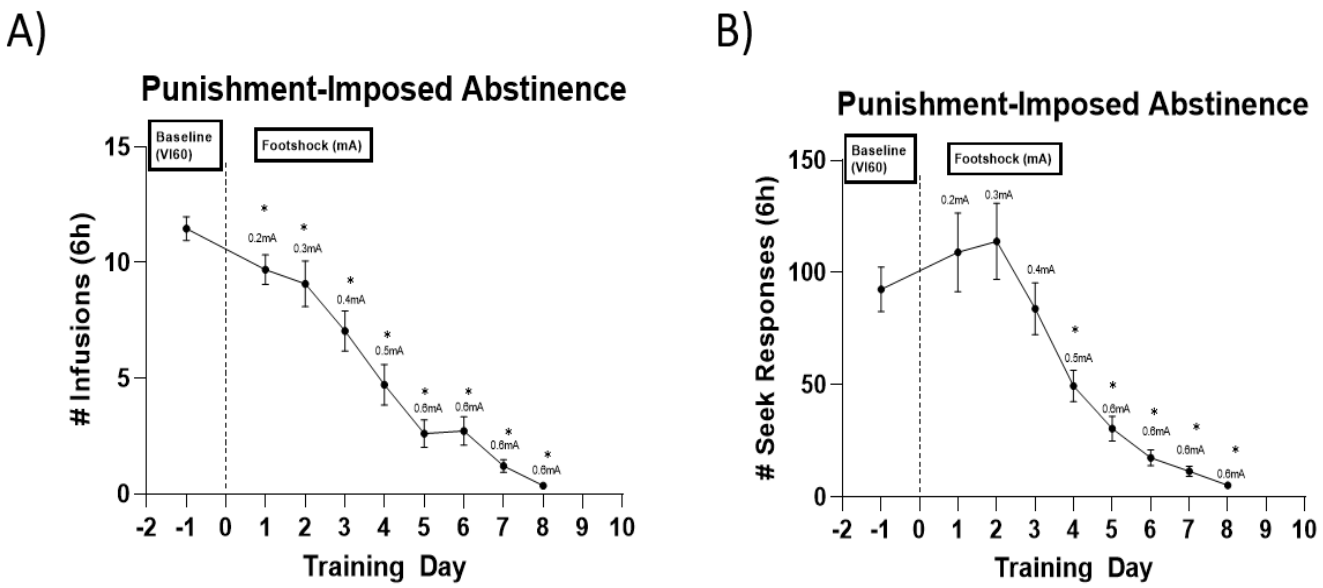

Figure 3. Mean (±SEM) number of (A) infusions (**p*'s < 0.02) and (B) seek lever presses (**p*'s < 0.003) for males on the average of the last three days on VI60 schedule (Baseline) followed by punishment-imposed abstinence (Footshock). The footshock intensity increased by 0.1 mA per training day (n= 52)

Experiment 1a. Restraint stress-induced relapse

Data Integrity: Two rats were removed from analyses, one due to illness, and another due to failure to meet self-administration criterion. In total, 18 rats were included in the analysis.

Heroin-seeking tests: Restraint stress did not induce an increase in heroin seeking while food deprivation resulted in a statistically significant relapse to heroin seeking (Fig. 4). A two-way ANOVA to compare the effect of restraint stress and food deprivation stress on heroin seeking showed a main effect of *treatment condition* ($F(1,17) = 18.93$, $p = .0004$, $\eta_p^2 = 0.53$) where treatment (stress) resulted in increased heroin seeking as compared with the control condition. There was also a main effect of *stressor type* ($F(1,17) = 11.34$, $p = .0037$, $\eta_p^2 = 0.4$), where food deprivation increased heroin seeking more than restraint stress. A statistically significant *treatment condition* x *stressor type* interaction effect was found ($F(1,17) = 18.95$, $p = .0004$, $\eta_p^2 = 0.53$) showing that only food deprivation stress (*MD*= 104.4, *SE of diff.*= 23.12), and not restraint stress (*MD* = 0.0, *SE of diff*= 6.38) significantly increased heroin seeking, as shown by Sidak's multiple comparisons test, $t(17) = 4.52$, $p = .0006$, $d = 1.46$ and $t(17) = 0.00$, $p > .999$, $d = 0$, respectively.

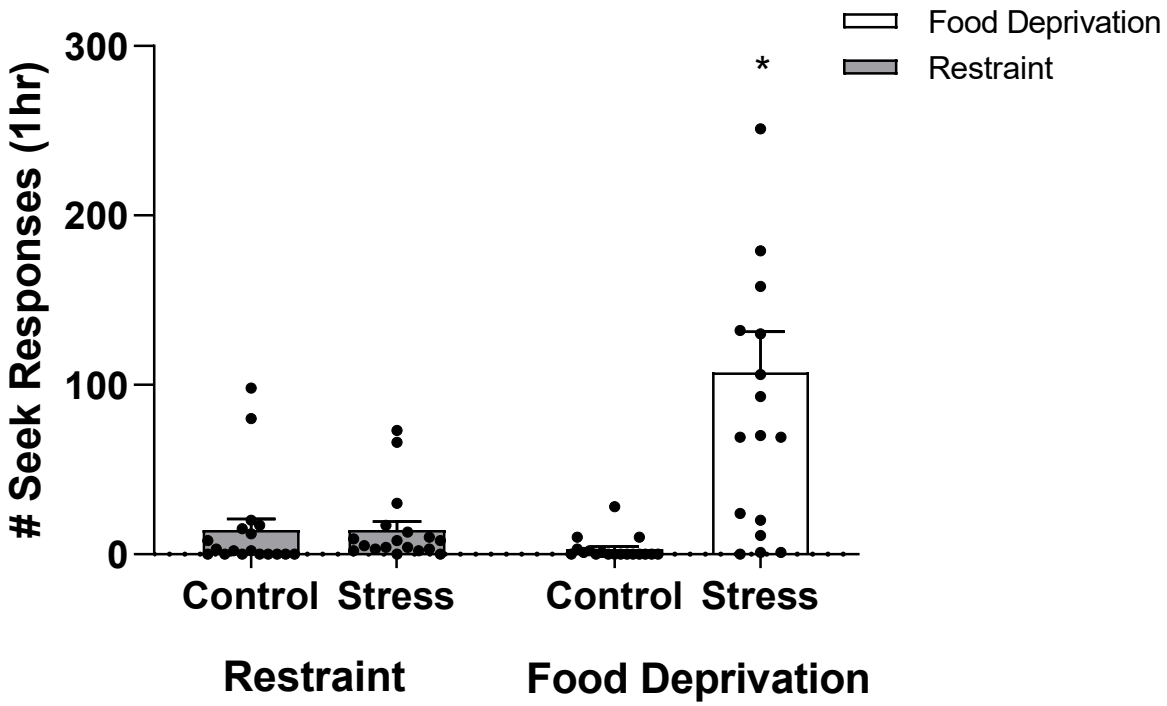

Figure 4. Mean (±SEM) number of seek lever presses for males during heroin-seeking tests following restraint and food deprivation stress between control and stress conditions (n=18); *p= .0006, d = 1.46

Experiment 1b. Forced swim stress-induced relapse.

Data Integrity: In total, two rats were removed from analyses due to illness. In total, 18 rats were included.

Heroin-seeking test: Both forced swim and food deprivation induced an increase in heroin seeking (Fig. 5). A main effect of *treatment condition* ($F(1,17) = 9.52$, $p = .0067$, $\eta_p^2 = 0.36$) showed that treatment (stress) resulted in increased heroin seeking as compared with controls. There was also a main effect of stress condition (forced swim vs. food deprivation; $F(1,17) = 13.26$, $p = .002$, $\eta_p^2 = 0.44$), with food deprivation increasing heroin seeking more than forced swim. A statistically significant *treatment condition* x *stressor type* interaction effect was found ($F(1,17) = 6.23$, $p = .0231$, $\eta_p^2 = 0.27$) showing that food deprivation (*MD*= 41.56, *SE of diff.*= 14.74) had a more robust effect on heroin seeking compared to forced swim stress (*MD* = 4.5, *SE of diff* = 2.1). Post-hoc tests did not find a significant difference between forced swim and control condition, while a significant difference was found for food deprivation compared to the sated condition, as shown by Sidak's multiple comparisons test, $t(17) = 2.82$, $p = .0235$, $d = 0.73$ and $t(17) = 2.142$, $p = 0.0917$, $d = 0.72$, respectively. However, considering the large effect size and the fact that we were specifically interested in the effect of forced swim a paired t-test was performed on the forced swim data separately. Forced swim stress (*M*= 6.9, *SEM*= 2.0) statistically significantly increased heroin seeking in comparison to the control condition (*M*= 2, *SEM*= 0.68; $t(17)= 2.33$, $p = .0324$, $d = 0.787$).

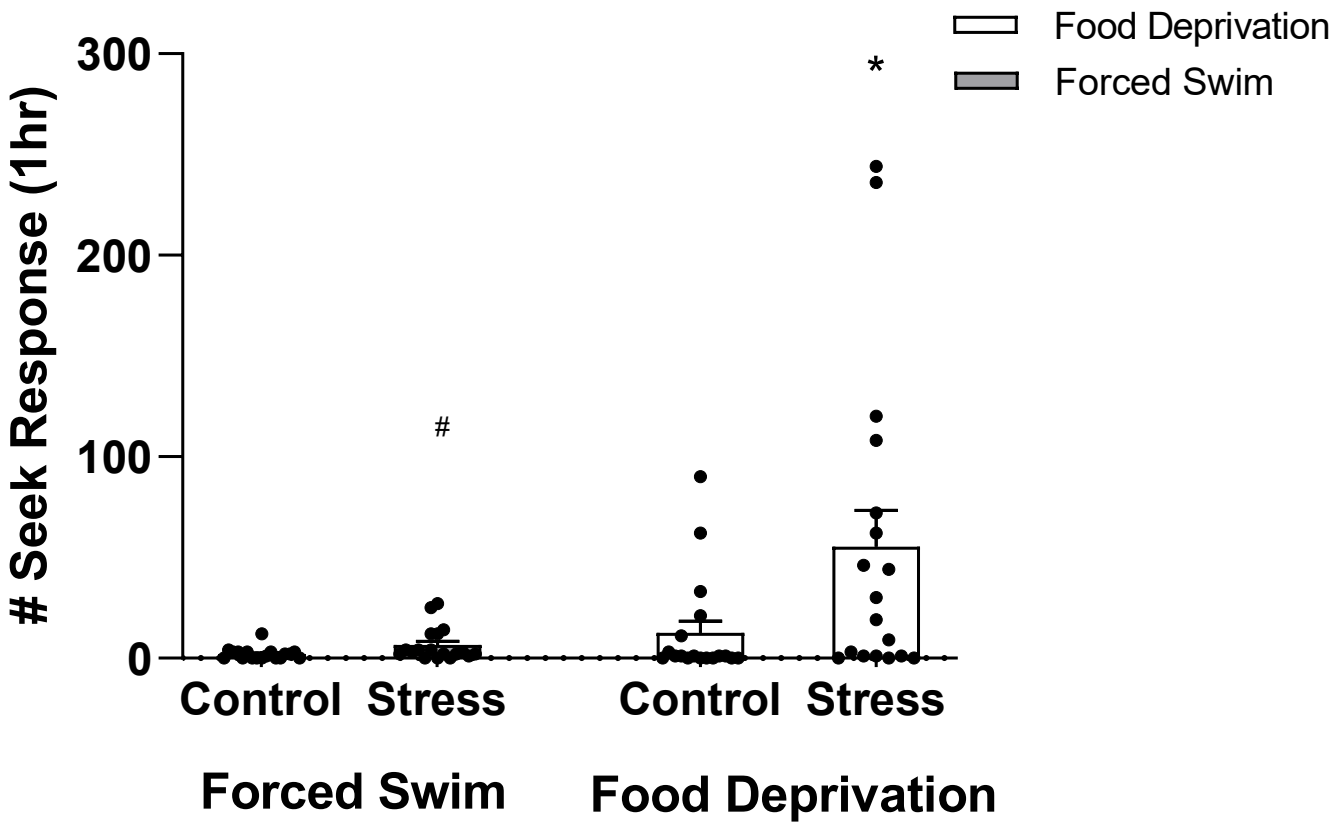

Figure 5. Mean (±SEM) number of seek lever presses for males during heroin-seeking tests following forced swim and food deprivation stress between control and stress conditions (n=18); **p* = .0235, *d* = 0.73 ; # *p*= 0.0324, *d* = 0.787 (see text for details)

Experiment 1c. Footshock stress-induced relapse

Data Integrity: Four rats were removed due to failure to meet self-administration criteria. In total, 16 rats were included in the analysis.

Heroin-Seeking Test: Footshock stress did not induce an increase in heroin seeking while food deprivation resulted in a statistically significant relapse to heroin seeking (Fig. 6). A two-way ANOVA to compare the effect of footshock and food deprivation stress on heroin seeking showed a main effect of *treatment condition* ($F(1,15)= 4.839$, $p= .0439$, $\eta_p^2 = .24$), where treatment (stress) resulted in increased heroin seeking as compared with the control condition. There was also a main effect of *stressor type* ($F(1,15) = 5.082$, $p = .0396$, $\eta_p^2 = 0.25$), where food deprivation increased heroin seeking more than footshock stress. No statistically significant interaction effect was found ($F(1,15) = 2.387$, $p = .143$, $\eta_p^2 = 0.14$).

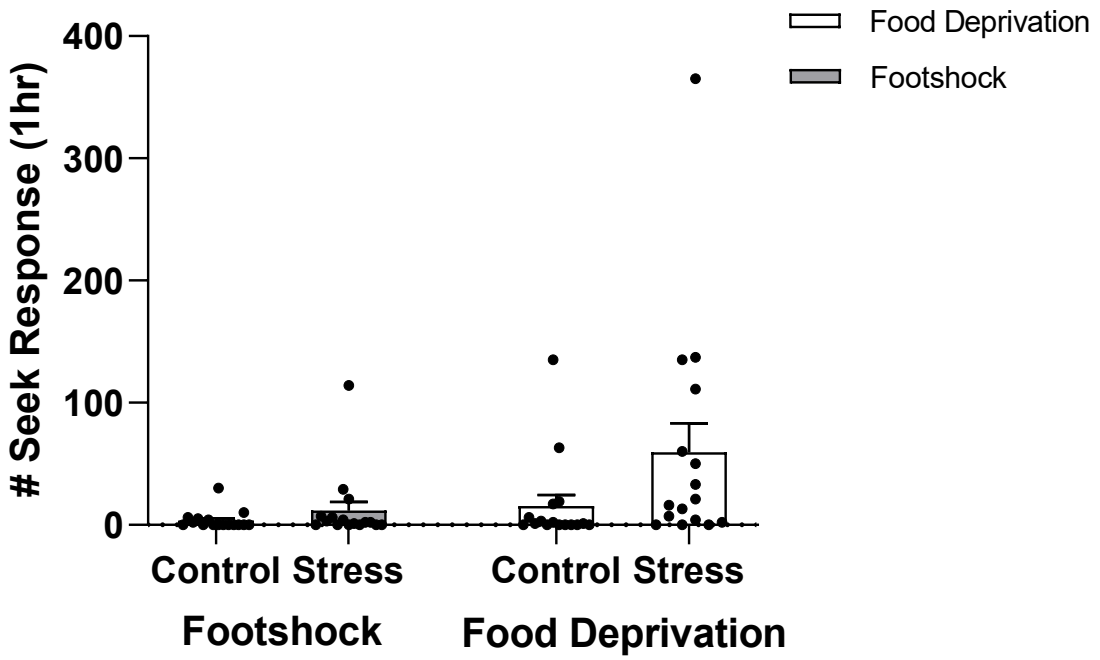

Figure 6. Mean (±SEM) number of seek lever presses for males during heroin-seeking tests following footshock and food deprivation stress between control and stress conditions (n=16)

Prescreenings

Only forced swim and food deprivation will be addressed here; for data pertaining to restraint and footshock, please see the appendix (Table 4).

Table 1. Regression analyses table: Individual characteristics as predictors of forced swim- or food-deprivation-induced relapse to heroin seeking in male rats. Bold font indicate significant effect.

Forced swim-induced relapse				
Percent Sucrose Preference	F(1,16) = 2.58	p = .128	r^2 = 0.139	B= -12.64, 95% CI [-29.33, 4.044]
Latency to Tail-Flick	F(1,16) = 1.85	p =.193	r^2 = 0.104	B= 2.94, 95% CI [-1.64, 7.53]
EPM: Time in the open arms	F(1,16) = 0.49	p = .495	r^2 = 0.030	B= 0.028, 95% CI [-0.057, 0.113]
EPM: Time freezing	F(1,16) = 0.03	p = .874	r^2 =0.002	B= -0.033, 95% CI [-0.47, 0.4]
EPM: Time grooming	F(1,16) =0.037	p = .85	r^2 =0.002	B= -0.027, 95% CI [-0.32, 0.27]
EPM: Distance travelled	F(1,16) =3.18	p = .094	r^2 = 0.166	B= 0.047, 95% CI [-0.0088, 0.1]
OF: Distance travelled	F(1,16) = 1.94	p = .183	r^2= 0.11	B= -0.17, 95% CI [-0.44, 0.091]
OF: Time freezing	F(1,16) = 3.91	p = .065	r^2= .197	B= 0.024, 95% CI [-0.002, 0.05]
OF: Time in center	**F(1,16) = 8.56**	***p* = .0099**	***r*^{2}=0.349**	***B*= 0.044, 95% CI [0.012, 0.076]**
OF: Fecal boli	F(1,16) = 0.194	p = .666	r^2 = .012	B = -0.27, 95% CI [-1.55, 1.02]

Food deprivation-induced relapse				
Percent Sucrose Preference	F(1,16) = 3.1	p = .098	r^2 = 0.162	B= -127.2, 95% CI [-280.5, 26.09]
Latency to Tail-Flick	F(1,16) =0.7	p =.417	r^2 = 0.0417	B= -17.37, 95% CI [-61.51 to 26.77]
EPM: Time in the open arms	F(1,16) = 0.15	p = .703	r^2 = 0.009	B= -0.146, 95% CI [-0.95, 0.66]
EPM: Time freezing	**F(1,16) = 4.95**	***p* = .0409**	***r*^{2} =0.236**	***B*= -3.73, 95% CI [-7.28, -0.17]**
EPM: Time grooming	F(1,16) =1.19	p = .291	r^2 =0.069	B= 1.37, 95% CI [-1.29, 4.02]
EPM: Distance travelled	F(1,16) = 2.46	p = .136	r^2= 0.133	B= -0.39, 95% CI [-0.91, 0.14]

OF: Distance travelled	F(1,16) = 0.43	p =.523	r^2= 0.026	B= 0.79, 95% CI [-1.78, 3.36]
OF: Time freezing	F(1,16) = 0.75	p =.4	r^2= 0.045	B= -0.107, 95% CI [-0.37, 0.16]
OF: Time in center	F(1,16) = 0.066	p= .801	r^2= 0.0041	B= 0.044, 95% CI [-0.32 0.41]
OF: Fecal boli	F(1,16) =1.62	p= .221	r^2=0.092	B= 6.9, 95% CI [-4.59, 18.39]

Experiment 2. Generalization of food deprivation-induced relapse to other acute stressors in female rats

Self-Administration training:

All rats (N=15) increased in seek lever presses as the variable interval increased across training days. Rats administered a consistent and reliable number of heroin infusions over the training days (Fig. 7).

Punishment phase: During punishment, rats decreased the number of seek lever presses and infusions as the footshock intensity increased over the 8 days (Fig. 8). Using a one-way repeated measures ANOVA, comparisons between the number of infusions and seek lever responses made throughout punishment days and baseline infusions and seek scores during self-administration (average of self-administration days under VI60) showed a significant change in the number of seek lever presses and infusions ($F(2.164, 30.3) = 7.731$, $p = .0015$, $\eta_p^2 = 0.356$, $F(2.361, 33.06) = 27.18$, $p < .0001$, $\eta_p^2 = 0.66$, respectively). A post hoc Dunnett's multiple comparisons test was run between the baseline and each of the 8 days of shock, which found that the baseline seeking was significantly different from seek scores of day 5 (shock 0.6 mA), day 6 (shock 0.6 mA), day 7 (shock 0.6 mA), and day 8 (shock 0.6 mA) of punishment-imposed abstinence (all; Fig. 9). Baseline infusions score was also significantly different from infusions on all 8 days of punishment-imposed abstinence (all p's < 0.02; Fig. 9).

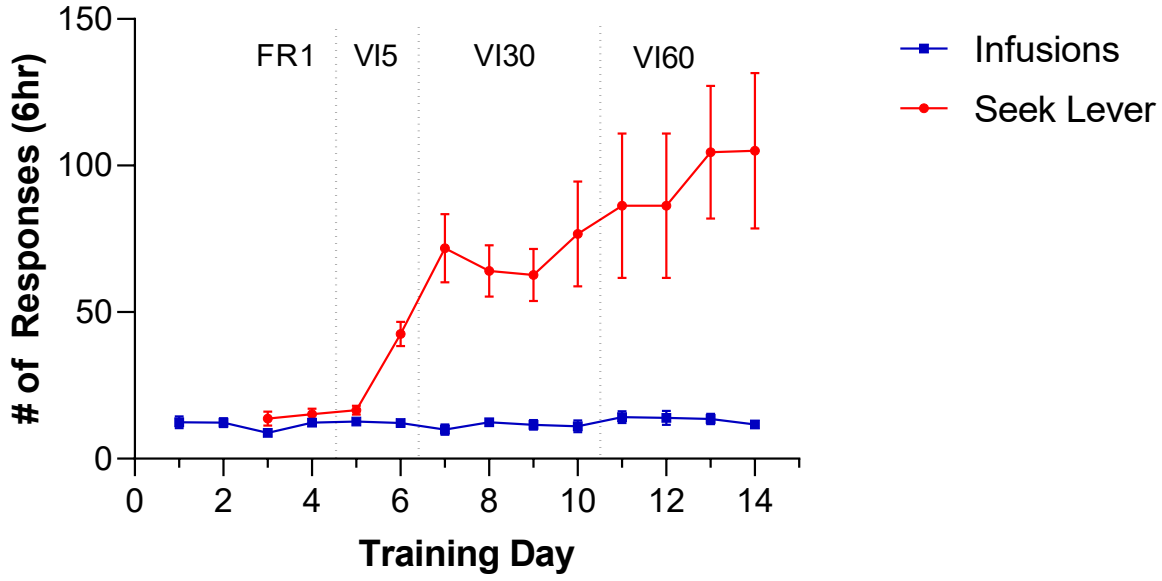

Figure 7. Mean (±SEM) number of seek lever presses (in red) and heroin infusions (in blue) throughout the duration of self-administration training separated by reinforcement schedule (FR1, VI5, VI30, VI60) in female rats (n= 15).

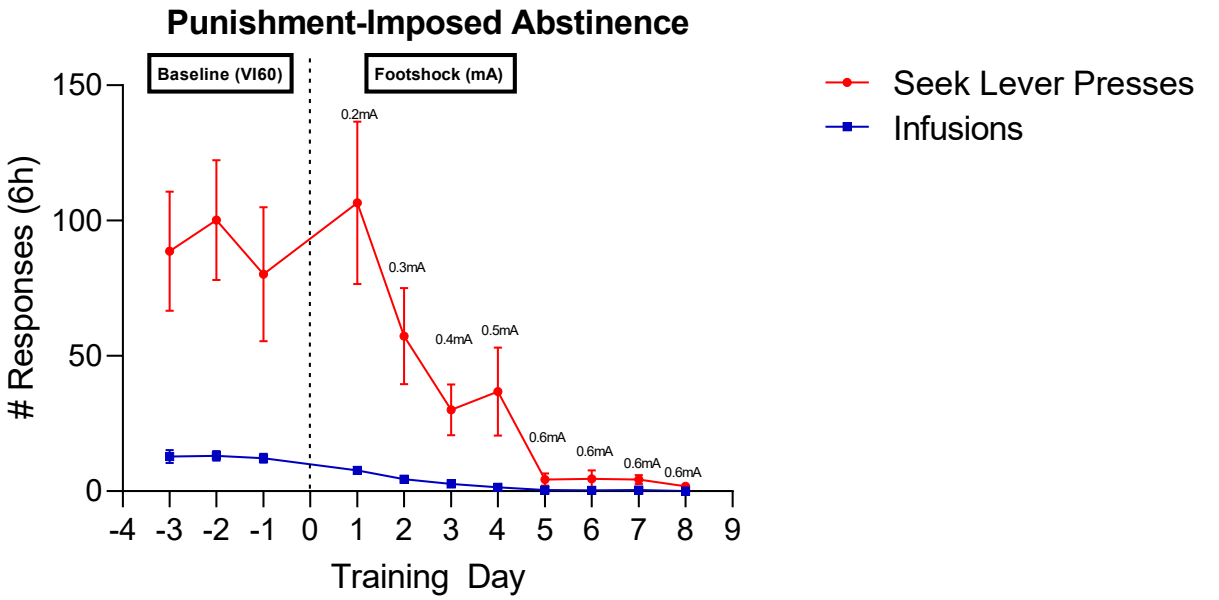

Figure 8. Mean (±SEM) number of seek lever presses and infusionsfor females on the last three days on VI60 schedule (Baseline) followed by punishment-imposed abstinence (Footshock). The footshock intensity increased by 0.1mA per training day (n= 15)

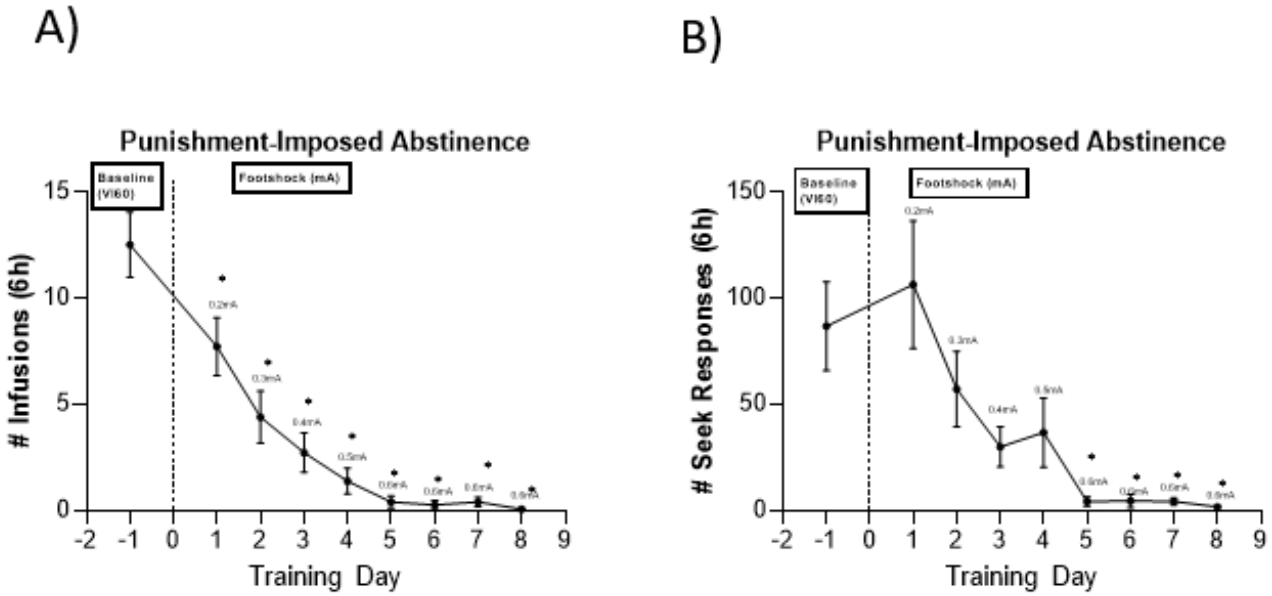

Figure 9. Mean (±SEM) number of (A) infusions (*p's < 0.02) and (B) seek lever presses (*p's < 0.02) for females on the average of the last three days on VI60 schedule (Baseline) followed by punishment-imposed abstinence (Footshock). The footshock intensity increased by 0.1mA per training day (n= 15)

Experiment 2a. Restraint stress-induced relapse.

Data integrity: Four rats were removed from analysis, two due to illness, one due to failure to meet punishment criteria, and one due to ROUT outlier analysis, leaving 6 rats.

Heroin-seeking tests: Restraint stress did not induce an increase in heroin seeking, while food deprivation did (Fig. 10). A two-way ANOVA showed a main effect of *treatment condition* ($F(1,5) = 12.97$, $p = .0101$, $\eta_p^2 = 0.76$) where treatment (stress) resulted in higher heroin seeking as compared with the no stress group. There was also a main effect of *stress condition* ($F(1,5) = 7.2$, $p = .0436$, $\eta_p^2 = 0.59$), where food deprivation increased heroin seeking more than restraint stress. A statistically significant *treatment condition* x *stress condition* interaction effect was found ($F(1,5) = 13.63$, $p = .0096$, $\eta_p^2 = 0.73$) supporting the conclusion that only food deprivation stress (*MD*= 62.83, *SE of diff.*= 13.06), and not restraint stress (*MD*= 5.68, *SE of diff*= 13.06) significantly increased heroin seeking, as shown by Sidak's multiple comparisons test, $t(5)= 5.74$, $p= .0045$, d= 2.12 and $t(5)= 0.518$, $p= 0.861$, d= 0.47, respectively.

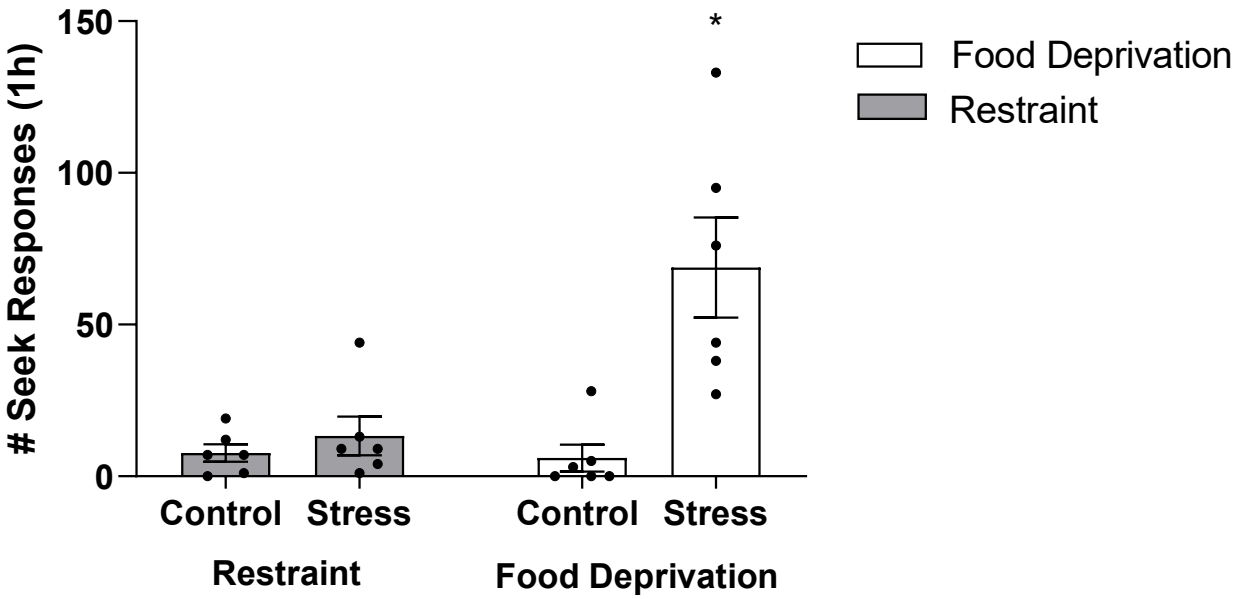

Figure 10. Mean (±SEM) number of seek lever presses for females during heroin-seeking tests following restraint and food deprivation stress between control and stress conditions (n=6). *p= 0.0045, d= 2.1

Experiment 2b. Forced swim stress-induced relapse.

Data Integrity: One rat was removed from analyses due to failure to meet abstinence criterion, leaving 9 rats.

Heroin-seeking tests: Forced swim stress was found to increase heroin seeking, as was food deprivation stress (Fig. 11). A two-way ANOVA showed a main effect of *treatment condition* ($F(1,8) = 9.381$, $p = .0155$, $\eta_p^2 = 0.54$) where treatment (stress) resulted in higher heroin seeking as compared with the no stress group. There was no statistically significant main effect of *stress condition* ($F(1, 8) = 5.219$, $p = .0517$, $\eta_p^2 = 0.395$). There was no statistically significant interaction effect ($F(1, 8) = 4.109$, $p = .0772$, $\eta_p^2 = 0.339$). Since the effect size seemed large, and a major aim of the study was to assess whether other acute stressors increase heroin seeking, a paired t-test was run for forced swim stress condition versus the control. Forced swim stress statistically significantly increased heroin seeking in comparison to the control condition ($t(8)= 2.432$, $p= .0411$, d=0.917). Food deprivation stress ($M= 56$, $SEM= 19.86$) statistically increased heroin seeking in comparison to the control condition ($M= 5.44$, $SEM= 3.11$) $t(8)= 2.765$, $p= 0.0245$, d= 1.186.

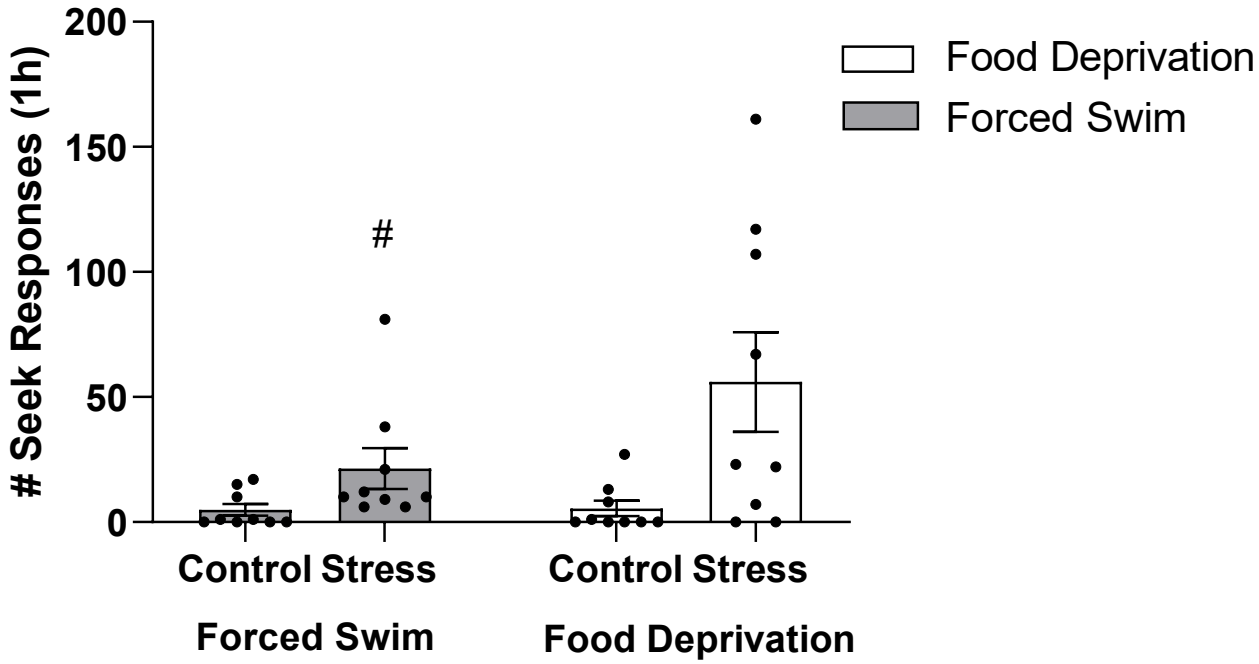

Figure 11. Mean (±SEM) number of seek lever presses for females during heroin-seeking tests following forced swim and food deprivation stress between control and stress conditions (n=8); # p = .0411, d=0.917

Prescreenings

Only forced swim and food deprivation will be addressed in the results section; for data pertaining to restraint, please see the appendix (Table 5).

Table 2. Regression analyses table: Individual characteristics as predictors of forced swim- or food-deprivation-induced relapse to heroin seeking in female rats.

Forced swim-induced relapse				
Percent Sucrose Preference	**F(1,7)= 6.454**	***p* = .039**	**r^2= 0.48**	***B*= 1.037, 95% CI -126.2, -4.524]**
Latency to Tail-Flick	F(1,7)= 0.254	*p* = .63	r^2 = 0.035	*B*= -12.5, 95% CI [-71.18, 46.18]
EPM: Time in the open arms	F(1,7)= 0.599	*p* = .464	r^2 = 0.0789	*B*= -0.065, 95% CI [-0.264, 0.134]
EPM: Time freezing	F(17)= 1.514	*p* = .258	r^2 = 178	*B*= 0.162, 95% CI [-0.149, 0.474]
EPM: Time grooming	F(1,7)= 0.683	*p* = .436	r^2 = 0.089	*B*=-0.157, 95% CI [-0.607, 0.292]
EPM: Distance travelled	F(1,7)= 0.292	*p* = .606	r^2 = 0.04	*B*=-0.73, 95% CI [-3.925, 2.465]
OF: Distance travelled	F(1,7)= 4.87	*p* = .063	r^2 = 0.41	*B*=-0.5, 95% CI [-1.04, 0.036]
OF: Time freezing	F(1,7)= 2.05	*p* = .195	r^2 = .227	*B*=0.037, 95% CI [-0.024, 0.099]
OF: Time in center	F(1,7)= 3.391	*p* = .108	r^2 = 0.326	*B*= -0.416, 95% CI [-0.951, 0.118]
OF: Fecal boli	F(1,7)= 2.434	*p* = .163	r^2 = 0.258	*B*= -5.418, 95% CI [-13.63, 2.795]
Food deprivation-induced relapse				
Percent Sucrose Preference	F(1,7)= 0.094	*p* =.768	r^2 = 0.013	*B*= 2.827, 95% CI -230.2, 177.3]
Latency to Tail-Flick	F(1,7)= 0.003	*p* = .958	r^2 = 0.000	*B*= 3.323, 95% CI [-141.8, 148.5]
EPM: Time in the open arms	F(1,7)= 0.988	*p* =.353	r^2 =0.124	*B*= -0.198, 95% CI [-0.67, 0.1274]
EPM: Time freezing	F(1,7)= 0.014	*p* = .908	r^2 = 0.002	*B*= -0.0422, 95% CI [-0.877, 0.792]
EPM: Time grooming	F(1,7)= 0.399	*p* = .762	r^2 = 0.014	*B*= -0.155, 95% CI [-1.288, 0.986]
EPM: Distance travelled	F(1,7)= 0.0206	*p* = .889	r^2 = 0.003	*B*= -0.48, 95% CI [-8.394, 7.43]
OF: Distance travelled	**F(1,7)= 5.973**	***p* = .045**	**r^2 = 0.46**	***B*= -1.289, 95% CI [-2.536, -0.042]**

OF: Time freezing	F(1,7)= 4.65	p = .068	r^2 = 0.399	B= 0.121, 95% CI [-0.012, 0.253]
OF: Time in center	F(1,7)= 3.243	p = .115	r^2 = 0.317	B= -0.997, 95% CI [-2.306, 0.312]
OF: Fecal boli	F(1,7)= 2.143	p =.187	r^2 = 0.234	B= -12.55, 95% CI [-32.83, 7.724]

Discussion

The study had three major aims: (1) to assess whether the effect of food deprivation would generalize to other acute stressors, primarily restraint, forced swim, and footshock stress, and result in relapse to heroin seeking following punishment-imposed abstinence in male rats; (2) to uncover the degree to which individual variation in personality characteristics predicted relapse following acute stress using a punishment-imposed abstinence paradigm; and (3) to see whether female rats respond similarly to punishment-imposed abstinence and whether acute stressors, such as food deprivation, restraint, and forced swim, increase heroin-seeking. Our hypotheses were that: (1) the effect of acute food deprivation stress would generalize to restraint, forced swim, and footshock stress, resulting in an increase in heroin seeking; (2) rats with higher anxiety-like behaviour, higher novelty-seeking behaviour, and higher sweet preference will have increased relapse to heroin-seeking following acute stress; and (3) female rats will show increased resistance to punishment during the punishment-imposed abstinence and show a greater propensity to relapse to heroin-seeking following acute stress.

Generalization of food deprivation effect on heroin relapse to other stressors

While acute food deprivation stress demonstrated a strong effect and increased heroin seeking, exposure to restraint stress in Experiment 1 did not, which was contrary to what we expected to find. One explanation for this could be that the intensity of the restraint stress employed was too low. There are four major ways to administer restraint: loose, tight, supine, and combination (Servatius et al., 2007). Loose restraint is often placed in a small chamber where the animal is not able to freely move, however some movement is still possible. Tight restraint takes this a step further and does not allow any movement at all. This is usually accomplished by using a jacket or harness to restrain or securing the animal's limbs while they

are laying prone. Supine restraint is a version of tight restraint where the animal is on its back and restrained. Combination restraint is when restraint stress is combined with another stressor. Each successive type of restraint listed here is more stressful than the last (Servatius et al., 2007). Our group employed a loose restraint method, and as such it may be that it was not stressful enough to induce a reaction. Additionally, the restraint tubes may have simply been not small enough, as it appears that the rats had enough room to turn around fully in the tubes. This may indicate that too much free movement was allowed. Further, some studies have administered restraint stress for longer periods than we did, for over 2 hours (Taslimi, Komaki, Sarihi, and Haghparast, 2019). In contrast, we used only 30-min periods in males. However, studies have shown an effect of restraint stress at 30 minutes (De Giovanni et al., 2016; Ribeiro Do Couto et al., 2006). In the literature, restraint stress has produced mixed results. Taslimi, Sarihi, and Haghparast (2018) found that restraint stress reinstated methamphetamine-seeking behaviour in rats using a conditioned place preference paradigm. Shalev, Highfield, Yap, and Shaham (2000) found that restraint stress administered outside the self-administration context did not reinstate heroin seeking. A study by Taslimi and colleagues (2018) found that acute and chronic restraint stress potentiates the effects of ineffective doses of methamphetamine and induces METH-induced CPP. This may suggest that restraint may be ineffective as a stressor on its own, but may work in-tandem with other relapse causes (like priming). It is important to note that the above studies did not use a voluntary abstinence procedure, and methodological differences (a conditioned-place preference paradigm, or a reinstatement model paradigm) between studies could contribute to the differences in findings.

When directly compared to food deprivation stress, forced swim stress did not statistically significantly increase heroin seeking, however it appeared to follow a significant

trend. When considered by itself, forced swim did statistically significantly increase heroin seeking. In the literature, forced swim stress reinstated drug-seeking behaviour in cocaine (Conrad et al., 2010), increased morphine-conditioned place preference (Farzinpour et al., Haghparast, 2019), escalated ethanol intake (Anderson, Lopez, and Becker, 2016), and increased nicotine-conditioned place preference (Jackson et al., 2013). Thus, we expected to find that forced swim stress augmented heroin-seeking. One reason for the lack of statistical significance when forced swim was directly compared to food deprivation stress may be the scaled difference in the amount each stressor increased heroin seeking. Food deprivation seemingly augmented heroin-seeking to much greater degrees, showing a much more robust effect of the stressor. Another possible reason for this could be the differences between the two batches of rats tested on the forced swim. We found that one batch responded much more strongly to forced swim than the other, with no apparent differences in health, heroin self-administration or punishment-imposed abstinence behaviour. Forced swim stress may be less robust than food deprivation due to the nature of the stressor- after removing the rats from the water, they were dried with a towel and immediately returned to the self-administration box to begin the 1-hour heroin-seeking test. During this time, we observed that rats spent large amounts of the initial time grooming during the self-administration tests, which does not occur during the heroin-seeking tests after food deprivation stress. Perhaps similar levels of responding following forced swim and acute food deprivation would be observed under longer heroin-seeking tests.

Tests with footshock stress also did not support our hypothesis and did not induce relapse to heroin seeking following punishment-imposed abstinence. This contrasts with Shalev et al. (2000) findings. They found that inescapable footshock stress for 5 or 15 minutes reinstates heroin seeking in the drug environment but not the non-drug environment. The effect was also

demonstrated in the reinstatement of cocaine (Erb, Shaham & Stewart, 1996), nicotine (Buczek et al., 1999), and alcohol (Le et al., 1998). The difference in findings could be due to methodological differences in the model, which may not be comparable (reinstatement is less translatable than voluntary abstinence). Another possible reason for the lack of effect could be that we used footshock as punishment during the punishment-imposed abstinence phase, and as such footshock may reinstate fear rather than cause an increase in heroin-seeking. While footshock in the punishment-imposed phase was contingent on seek response and in the stress phase it was non-contingent and unavoidable, the association between seek response and punishment could have prevented relapse to heroin seeking. For example, after extinction, re-exposure to the US can reinstate conditioned responding to a conditioned stimulus (CS; Bouton & Bolles, 1979; LeCocq, Sun, & Chaudhri, 2022). Re-exposure to an aversive unconditioned stimulus (US) reinstates many conditioned responses, including the suppression of an operant response (Rescorla & Heth, 1975; Bouton & Bolles, 1979; LeCocq, Sun, & Chaudhri, 2022). In our case, presentation of footshock (US) as a stressor, could have resulted in the strengthening of the suppression of the heroin-seeking response in the context of the heroin-seeking test, when the seeking-lever (CS) became available.

These findings suggest that acute food deprivation may indeed be a unique acute stressor in its robustness. Even with forced swim augmenting heroin-seeking, acute food deprivation increased relapse behaviour to a much larger degree.

Generalization of stress-induced relapse in males to female rats

There are biological and behavioural differences between males and females. Sex differences in addiction research are understudied; however, in the studies including sex differences, findings have been mixed depending on the drug class, addiction phase and model.

In most drug classes, female rats were more susceptible to stress and cue-induced reinstatement (Anker & Carroll, 2010; Hudson & Stamp, 2011).

Orsini et al. (2016) found that, when tested under a "Risky Decision making Task" (RDT) where male and female rats chose between a smaller, less risky food reward and a large, risky food reward where there was an increasing probability of footshock punishment, female rats were more risk averse. Interestingly, in later studies, Orsini et al. (2020), did not find any sex differences in choice of the large, risky reward. They do claim that this could be the result of experimental design which was structured to detect individual rather than group sex differences. In Orsini et al. (2016), shock intensity was identical across male and females, whereas in Orsini et al. (2020), shocks were separately adjusted for each sex to maximize the range of individual variation in risk preference. Sutton et al. (2021) found that male and female rats showed similar levels of resurgence of alcohol seeking following abstinence induced by punishment co-administered with an alternative non-drug reward, when the alternative reward was removed. However, following punishment without an alternative reward, removal of the punishment resulted in relapse to alcohol seeking in females but not in males. Thus, females may be faster than males to return to a previously punished response (Sutton et al., 2021). They also found that alcohol seeking of females was less suppressed by exposure to punishment alone than in tandem with an alternative non-drug reward. In our study, both male and female rats decreased heroin-seeking over the 8 days of punishment, however the area under the punishment curve was much smaller for females (M=191.5, SEM= 40.35) than for male rats (M=365.5, SEM= 42.23), suggesting females are more susceptible to punishment (Table 3). This aligns with Orsini et al. (2016) who found that females were more risk averse, and with Chowdhury et al. (2019), who demonstrated that that females were faster in punishment-avoidance learning, and more sensitive

to probabilistic punishment. In our study, punishment was probabilistic, in that punishment only occurred on 30% of trials, and thus may be a reason females were more sensitive. Our results do, however, differ with Sutton et al. (2021) and this could be due to differences in methodology and drug class. Sutton et al. (2021) did not use a seek-take procedure, and instead used only a take lever that, during punishment, produced both the alcohol reward on a VI5 schedule and a mild (0.5 mA) footshock on every response. In our study, punishment occurred on the seek-lever during 30% of trials, and drug taking was not punished, so it is possible that females are more resistant to punishment only under certain conditions, e.g. when drug taking is punished on every trial (i.e. not probabilistic), or perhaps with different drug classes.

In terms of sex differences between males and females on relapse, they could not be directly compared due to methodological differences. We reduced forced swim time from 20 minutes for males to 4 minutes for females. This was due to unexpected differences in swimming abilities between males and females, with females being noticeably weaker swimmers. Restraint time was increased from 30 minutes in males to 1 hour in females due to having a smaller body weight. We thought that since the tubes were the same size, increasing the time might offset the extra room that females had to move around. However, when analyzed separately, both male and female rats showed a significant increase in heroin seeking following food deprivation stress. Following restraint stress, when analyzed separately, male, and female rats did not increase heroin-seeking. When looking at forced swim, both male and female rats did increase heroin seeking with high effect sizes (Fig. 12). These results contradict findings on stress-induced reinstatement, where females were found to relapse at higher intensity (Anker & Carroll, 2010). The discrepancy could be explained by the use of the reinstatement model, as extinction models and abstinence models use different neural pathways (Fuchs, Branham, & See, 2006). Another

explanation could be the use of yohimbine as a “pharmacological stressor” by Anker & Carroll (2010). It was suggested that administration of yohimbine, an anxiogenic drug, results in general activation rather than relapse to drug seeking (Chen et al., 2015).

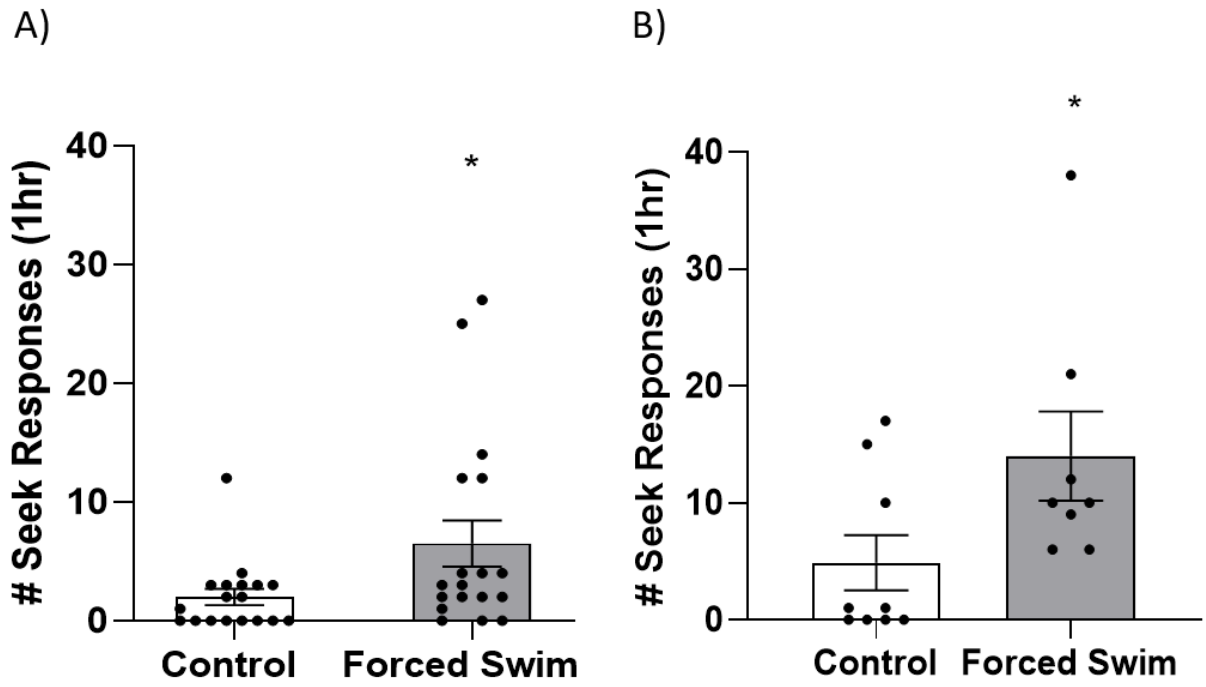

Figure 12. Mean (±SEM) number of seek lever presses during heroin-seeking tests following forced swim stress between control and stress conditions for (A) males (n= 18) *p= 0.047, d= 0.73 and; (B) females (n= 8) *p= 0.0441, d= 0.917

In this study, we decided to not analyze sex differences in terms of stage of the estrus cycle, despite tracking it, due to the small number of animals in each stage of estrus at any given time point. In the future, with larger sample sizes, this may be an interesting avenue to consider to see how hormonal fluctuations may relate to sensitivity to punishment and relapse behaviour.

Individual traits as predictors of stress-induced relapse

The relationships between personality traits and relapse behaviour were largely non-significant in both male and female rats. However, some interesting relationships were observed with the level of heroin seeking during the relapse tests, even when formal "relapse" was not established. In males, time spent in the center of the open field apparatus positively predicted heroin-seeking following forced swim stress (where significant relapse was observed) and footshock, suggesting that the less anxious the animal, the higher the propensity to seek-heroin following those stressors. Time spent freezing (where more time freezing indicates increased anxiety-like behaviour) in the elevated plus maze inversely predicted heroin-seeking following food deprivation stress and footshock stress, meaning the more time spent freezing, the less the animal sought heroin following stress. These findings suggest that the less anxious the animal is, the more the animal will seek heroin following stress. In direct contrast to this, time spent freezing in the open field positively predicted heroin-seeking following footshock stress only, suggesting a more anxious animal will seek more heroin following stress. The only other significant association in the male data was an inverse correlation for latency to tail-flick and heroin seeking following restraint stress, suggesting rats with higher pain sensitivity showed increased heroin-seeking behaviour following restraint stress only.

In female rats, percent sucrose preference was positively correlated with forced swim stress, meaning that the greater the sweet preference, or the greater the animal's proclivity for

reward, the more animals sought heroin following forced swim stress. Distance travelled in the OF inversely predicted heroin seeking following food deprivation stress. This indicates that the more exploratory the animal, the lower the propensity to relapse to heroin-seeking following food deprivation stress. Finally, time spent freezing in the elevated plus-maze and open field arena (whereby more time freezing suggests increased anxiety-like behaviour) was positively correlated with heroin-seeking following restraint stress (See Appendix, Table 4).

When interpreting these results, it is important to remember that restraint and footshock stress did not induce relapse to heroin-seeking. Therefore, the correlation between personality factors and heroin-seeking (see Appendix, Table 4) following these stressors should be interpreted cautiously. Possible reasons for such contrasting and underwhelming findings might be found in the debate surrounding animal behavioural testing. According to Carter et al. (2013), one of the criticisms of animal personality testing is that there are many tests for one trait. This is because tests are not always comparable, and there is a lack of standardization amongst tests for measuring the same behaviour. Tests may measure entirely different constructs. The test context is often intricately linked with what is assessed. For example, with the open field arena, whether the animal was introduced by force (via placing it in the environment without the ability to escape) or whether it was offered free exploration with access to a refuge (like a home cage) may influence what trait is truly being measured. Free open field tests are thought to measure voluntary exploration, whereas forced may measure fear or anxiety (Carter et al., 2013). With the conflicting results among traits we have seen, some tests we chose may not measure the same aspects of a construct or the construct entirely. Another issue, according to Carter et al. (2013), is that one test might measure several different traits. This is because the test can be influenced by and therefore measure multiple traits. It is hard to differentiate which trait is truly being

measured and what kind of interaction might occur. This is relevant for the elevated plus maze and open field test; we used both to assess various traits (anxiety and exploration), with the open field test, in particular, measuring various constructs such as anxiety, locomotion, exploratory behaviour, novelty-seeking, and more. Individual differences in traits may predict relapse; it is just that these traits may not be captured by the behavioural tests employed.

Methodological considerations

The behavioural tests used here as reflection of personal traits were not validated in our laboratory. However, we decided on these tests because these are the accepted standard tests most often used in the field (Hogg, 1996; Seibenhener, & Wooten, 2015; Hoffman, 2016). Another point to consider is the difference in the parameters of the acute stressors between males and females. As discussed, the methodology of restraint and forced swim tests were changed when run in female rats compared to males. Additionally, the order of acute stressors and food deprivation stress was changed between male and female runs. Initially, in male runs, acute stressors were administered before food deprivation. For females, food deprivation was administered first, followed by acute stress. Consequently, it is difficult to directly compare the results of the stress-induced relapse. In the future, a direct comparison would strengthen the results. Additionally, the within-subjects design we used could have influenced the outcome of the relapse tests. Four relapse tests were run on each subject across the course of stress-induced relapse. Although the stress conditions (control and stress) were counterbalanced, each rat underwent two stressors (an acute stressor and food deprivation stress). In each test, rats underwent a relapse test under extinction conditions. Because of this, it is possible that rats' heroin-seeking behaviour was extinguished, leading to a less robust response to stress on the second administration of a stressor. The opposite could also be true, with the rats “primed”

following the first stressor exposure, causing an increase in the stress response on the second stressor more than we would typically see. Within-subjects designs are beneficial in some important ways, however: these designs minimize the number of subjects required. Further, subjects can serve as their own control and thus reduce the effects of individual differences on the outcome. Finally, experimental stressors are not entirely reflective of the human condition, as physical stressors are less frequently experienced than emotionally based stressors (such as divorce, financial struggles, or loss of a job). However, food deprivation is a stressor commonly experienced by humans, especially in drug-using populations, as drug users commonly allocate resources towards obtaining their drug of choice rather than food.

Future direction

It would be interesting to investigate impulsivity as a predictor of drug relapse, considering all the literature pointing to this link (Bowden-Jones et al., 2005; Paulus, Tapert, & Schuckit, 2005; Economidou et al., 2009). Another interesting avenue to explore is the effects of sex hormones on drug-seeking. Men and women have been shown to differ in their drug-use behaviours and isolating the effects of sex hormones may be a good avenue to investigate using a punishment-imposed abstinence model. For example, estrogen and progesterone have been shown to have opposite impact on drug-associated behaviours (Quinones-Jenab & Jenab, 2010; Anker & Carroll, 2011). Tracking the estrus cycle will continue to be important to evaluate differences in behaviour within each stage of the addiction cycle, specifically during punishment learning and stress relapse. Further, actively controlling the cycle may allow for the elucidation of causal links.

Conclusion

The punishment-imposed abstinence and stress-induced relapse model was validated in male and female rats, using the seek-take heroin self-administration procedure. Females were more sensitive to punishment, suggesting that contrary to expectations, female rats are not more punishment-resistant or more likely to relapse to heroin-seeking under a punishment-imposed model. The increase in heroin-seeking seen following acute food deprivation generalized to forced-swim stress only. However, forced-swim augmented heroin-seeking to a much lesser degree, suggesting unique properties of acute food deprivation stress. Finally, individual variation in trait measures of anxiety, sucrose-preference and novelty-seeking did not predict stress-induced relapse in a unified way. In conclusion, our study expands upon relapse to acute stress research using a punishment-imposed abstinence model and demonstrates that relapse behaviour may be stressor and/or drug-class specific. It further contributes to sex differences research in the field of addiction, highlighting the importance in considering drug class, model, and sex when interpreting drug use research.

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APPENDIX

Table 1.

Reduction in Heroin Seeking Lever Response following Punishment-Imposed Abstinence for Male Rats

		Seek Lever presses		
Training day	Schedule	*M*	*SEM*	*N*
-3	VI60	97.269	10.7	52
-2	VI60	79.04	8.67	51
-1	VI60	98.14	12.48	52
1	Shockers 0.2 mA	109.12	17.56	52
2	Shockers 0.3 mA	113.89	17.03	52
3	Shockers 0.4 mA	83.92	11.55	52
4	Shockers 0.5 mA	49.52	7.03	52
5	Shockers 0.6 mA	30.56	5.49	52
6	Shockers 0.6 mA	17.54	3.49	52
7	Shockers 0.6 mA	11.58	2.23	52
8	Shockers 0.6 mA	5.37	1.23	52

Table 2

Reduction in Heroin Seeking Lever Response following Punishment-Imposed Abstinence for Female Rats

		Seek Lever presses		
Training day	Schedule	*M*	*SEM*	*N*
-3	VI60	88.71	22.01	14
-2	VI60	98.94	20.46	15
-1	VI60	80.14	24.84	14
1	Shockers 0.2 mA	106.53	29.98	15
2	Shockers 0.3 mA	57.27	17.75	15
3	Shockers 0.4 mA	30	9.49	15
4	Shockers 0.5 mA	36.8	16.23	15
5	Shockers 0.6 mA	4.27	2.33	15
6	Shockers 0.6 mA	4.6	3.2	15
7	Shockers 0.6 mA	4.33	1.62	15
8	Shockers 0.6 mA	1.87	0.87	15

Table 3.

Total Area Under the Curve for Seek Lever Presses During Punishment-Imposed Abstinence: Males and females

Sex	*M*	*SEM*
Male	364.5	42.23
Female	191.5	40.35

Note. Female N = 15, Male N = 52.

Table 4.

Total Area Under the Curve for Seek Lever Presses During Punishment-Imposed Abstinence: Males and females

Sex	*M*	*SEM*
Male	32.9	3.51
Female	16.43	3.99

Note. Female N = 15, Male N = 52.

Table 5.

Regression analyses table: Individual characteristics as predictors of restraint- or footshock - induced relapse to heroin seeking in male rats.

Restraint-induced relapse				
Percent Sucrose Preference	F(1,16) = 2.355	p = .144	r^2= 0.128	B= 23.74, 95% CI [-9.052, 56.53]
Latency to Tail-Flick	F(1,16) = 4.672	p = .046	r^2= 0.226	B= -19.46, 95% CI [-38.54, -0.375]
EPM: Time in the open arms	F(1,16) = 0.114	p = .74	r^2= 0.007	B= -0.02, 95% CI [-0.145, 0.105]
EPM: Time freezing	F(1,16) = 0.774	p = .392	r^2= 0.0461	B= -0.033, 95% CI [-0.112, 0.046]
EPM: Time grooming	F(1,16) = 4.027	p = .062	r^2= 0.201	B= -0.438, 95% CI [-0.901, 0.025]
EPM: Distance travelled	F(1,16) = 0.346	p = .565	r^2= 0.021	B= -0.34, 95% CI [-1.566, 0.886]
OF: Distance travelled	F(1,16) = 0.858	p = .368	r^2= 0.051	B= 0.28, 95% CI [-0.361, 0.92]
OF: Time freezing	F(1,16) = 1.984	p = .178	r^2= 0.11	B= -0.044, 95% CI [-0.109, 0.022]
OF: Time in center	F(1,16) = 0.075	p = .787	r^2= 0.005	B= -0.051, 95% CI [-0.446, 0.344]
OF: Fecal boli	F(1,16) = 0.558	p = .466	r^2= 0.034	B= -1.038, 95% CI [-3.987, 1.91]
Footshock-induced relapse				
Percent Sucrose Preference	F(1,14) = 0.279	p = .606	r^2= 0.02	B= -14.55, 95% CI [-73.62, 44.52]
Latency to Tail-Flick	F(1,14) = 2.094	p = .17	r^2= 0.13	B= -6.204, 95% CI [-15.4, 2.99]
EPM: Time in the open arms	F(1,14) = 0.495	p = .493	r^2= 0.0342	B= -0.047, 95% CI [-0.188, 0.095]
EPM: Time freezing	F(1,14) = 0.664	p = .0453	r^2= 0.045	B= -0.033, 95% CI [-0.121, 0.055]
EPM: Time grooming	F(1,14) = 0.505	p = .489	r^2= 0.035	B= -0.402, 95% CI [-1.615, 0.811]
EPM: Distance travelled	F(1,14) = 0.44	p = .518	r^2= 0.031	B= -0.787, 95% CI [-3.334, 1.759]
OF: Distance travelled	F(1,14) = 1.887	p = .191	r^2= 0.119	B= -0.294, 95% CI [-0.752, 0.165]
OF: Time freezing	F(1,14) = 14.42	p = .002	r^2= 0.507	B= 0.092, 95% CI [0.04, 0.143]
OF: Time in center	F(1,14) = 27.02	p = .0001	r^2= 0.659	B= 60.97, 95% CI [0.059, 0.143]
OF: Fecal boli	F(1,14) = 2.264	p = .155	r^2= 0.139	B= 2.726, 95% CI [-1.159, 6.611]

Table 6.

Regression analyses table: Individual characteristics as predictors of restraint-induced relapse to heroin seeking in female rats.

Restraint-induced relapse				
Percent Sucrose Preference	F(1,4)= 3.25	p = .146	r^2= 0.448	B= -59.88, 95% CI [-151.1, 32.35]
Latency to Tail-Flick	F(1,4)= 1.34	p = .311	r^2= 0.251	B= -58.41, 95% CI [-198.5, 81.68]
EPM: Time in the open arms	F(1,4)= 1.98	p = .232	r^2= 0.332	B= -0.175, 95% CI [-0.519, 0.17]
EPM: Time freezing	F(1,4)= 38.2	p = .0035	r^2= 0.905	B= 0.528, 95% CI [0.291, 0.766]
EPM: Time grooming	F(1,4)= -0.554	p = .142	r^2= 0.455	B= -0.554, 95% CI [-1.396, 0.288]
EPM: Distance travelled	F(1,4)= 1.025	p = .386	r^2= 0.255	B= -0.493, 95% CI [-2.041, 1.056]
OF: Distance travelled	F(1,4)= 2.247	p = .208	r^2= 0.36	B= -0.294, 95% CI [-0.838, 0.251]
OF: Time freezing	F(1,4)= 13.25	p = .022	r^2= 0.768	B= 0.124, 95% CI [0.03, 0.219]
OF: Time in center	F(1,4)= 4.678	p = .097	r^2= 0.539	B= -0.409, 95% CI [-0.933, 0.116]
OF: Fecal boli	F(1,4)= 0.45	p = .539	r^2= 0.101	B= -2.513, 95% CI [-12.91, 7.886]