Conditioned Aversive Responses Produced by Delayed, but not Immediate, Exposure to Cocaine in Male Sprague-Dawley Rats

by

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ABSTRACT

CONDITIONED AVERSIVE RESPONSES PRODUCED BY DELAYED, BUT NOT IMMEDIATE, EXPOSURE TO COCAINE IN MALE SPRAGUE-DAWLEY RATS

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Cocaine abuse is accompanied by the emergence of negative affect such as dysphoria irritability and anhedonia. Cues associated with cocaine are rapidly learned by animals and are capable of eliciting a negative affective state that can trigger relapse, indicating a critical role for associative mechanisms in the regulation of drug seeking. Using the taste reactivity test, the conditions under which a taste paired with delayed cocaine is able to elicit a conditionally aversive state was examined. It is demonstrated that delayed cocaine produces conditioned gaping when saccharin is paired with either a 10- or 30-min delay, whereas immediate cocaine does not result in aversive behaviours. Morphine also produces conditioned gaping in the 10 min procedure. Rats who received delayed cocaine-paired saccharin exhibit increased anxiety-like behaviours. Saccharin needed to be administered intermittently throughout the delay in order to produce the conditioned aversive state. Pre-treatment with ondansetron, SCH 23390 or OGly interfered with the establishment of conditioned gaping to saccharin paired with delayed cocaine, but only during early trials.
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CHAPTER 1

General Introduction

Cocaine addiction is a disorder characterized by chronic relapse, causing significant concern for the treatment of this disorder (Hymen et al. 2006). The persistence of this disorder despite therapeutic treatment and long periods of abstinence can be partially attributed to cocaine’s reinforcing effects, such as euphoria and increased energy. However, of greater significance is the long-lasting associations that are made between cocaine and cocaine-associated cues. In fact, cocaine users who attempt to abstain often report being able to endure anhedonia and dysphoria associated with the cessation of cocaine until presented with conditioned cocaine cues (Gawin 1991). This indicates that cues play a fundamental role in the persistence of this disorder. Both humans and animals rapidly form drug-cue associations for stimuli that are temporally paired with the rewarding effects of drugs of abuse. Specifically, associations formed between cocaine and cocaine related-cues are often long-lasting and contribute to the persistence of addiction, as these cues predict the availability of the drug, induce feeling of craving and withdrawal, and consequently have the ability to motivate drug seeking and promote the occurrence of relapse (Hymen et al. 2006; O’Brien et al. 1998). Cocaine related cues can be environmental, such as a location, a particular object or a person, or they can be related to an individual’s internal state, like intoxication from another drug, mood or stressful events (Childress et al. 1993; Sinha, Catapano and O’Malley 1999; Gawin 1991).

Cocaine conditioned cues are known to produce intense craving. Although the definition of craving is ambiguous, it is often defined as a physiological withdrawal symptom that occurs as a result of the desire for the euphoric effects of a particular rewarding drug (Gawin 1991).
Craving is described as either a desire to use a drug, desire for the positive effects of the drug or behavioural intent to use the drug (Sayette et al. 2000). Despite the definitional nuances, it is agreed upon that there is a relationship between drug addiction and craving, and that drug related cues can result in the emergence of this craving (Sayette et al. 2000; Ehrman et al. 1992; Gawin 1991). Negative affect also plays a prominent role in craving and relapse. Specifically, cocaine use is often accompanied by the emergence of negative affective symptoms such as dysphoria, irritability, anhedonia and anxiety (e.g., Koob and LeMoal, 1997; Solomon and Corbit, 1974; Wheeler et al. 2008), and it has been shown that the greater these negative feelings, the greater the subjective euphoric effect of subsequent cocaine administration in humans (Newton et al. 2003; Sofuoglu et al. 2003). A negative affective state can also become associated with drug-related cues and can contribute to feelings of craving and subsequent drug seeking behaviours. Recent research has used the taste reactivity (TR) paradigm to examine the potential of a taste stimulus to act as drug-related cue, in order to directly measure negative affect by demonstrating that a taste cue paired with delayed access to cocaine elicits a conditioned aversive state that is quantifiable and predicts greater subsequent cocaine intake. (Wheeler et al. 2008; Wheeler et al. 2011).

*Taste Reactivity, Conditioned Taste Aversion and Conditioned Taste Avoidance*

The TR test (Grill and Norgren 1978a) allows researchers to determine the hedonic valence of a taste stimulus based on the behavioural responses elicited by the taste (Berridge 2000). When rats are intraorally infused with a palatable substance, such as saccharin, they will exhibit stereotyped ingestive responses such as tongue protrusions, indicating that they like the
flavour. Contrarily, when being intraorally infused an aversive flavour, such as the bitter solution quinine, rats display stereotyped aversive behaviours such as chin rubbing, paw treading and, most notably, gaping. The gaping response is characterised by a wide triangular opening of the mouth, with retraction of the corners of the mouth which exposes the rats’ lower incisors and this behaviour indicates an aversion to the flavour being administered (Grill and Norgren 1978a). In addition to displaying the intrinsic reaction a rat has to a taste stimulus, the TR test also gives an indication of any hedonic shifts that may occur. For example, when a naturally palatable solution, such as saccharin, is paired with an aversive drug, such as lithium chloride (LiCl), an association forms between the two resulting in conditioned aversion to the palatable flavour (Garcia and Koelling 1966). This results in rats displaying conditioned aversive responses to the previously palatable flavour, similar to those seen when administering aversive flavours. Therefore, this paradigm is able to assess conditioned changes in affect that are dissociable from simple ingestive behaviours.

In addition to producing taste aversion, LiCl and other aversive drugs also produce conditioned taste avoidance (CTA), meaning rats will avoid a flavour that has been previously paired with an aversive substance (Parker 1995). Interestingly, many rewarding drugs that are reliably self-administered or are capable of producing a conditioned place preference can also cause taste avoidance in rats (Parker 1993). When compared, rewarding drugs often show a conditioned taste avoidance equivalent to that of LiCl without producing any aversive TR behaviours characteristic of LiCl-paired flavours (Parker 1995). Cocaine is one such drug that is capable of producing a CTA (e.g. Parker 1993; Ferrari et al. 1991; Jones et al. 2010). Additionally, when comparing routes of administration, intraperitoneal (ip) cocaine produces a
relatively weak CTA, whereas subcutaneous (sc) cocaine is capable of producing a more robust avoidance (Ferrari et al. 1991). This is likely due to the different rates of absorption between the routes of administration, with ip cocaine producing shorter lasting effects than sc cocaine (Ferrari et al. 1991). Subsequent research has also shown that greater avoidance of a flavour that has been paired with cocaine results in greater cocaine self-administration (Grigson and Twinning 2002).

Although many experiments conclude that avoidance of a flavour implies an aversion to that flavour, this may not be true. Rats and mice are species that are not capable of vomiting, therefore any change in physiological state, whether good or bad, following ingestion of a novel flavour will result in them later avoiding that flavour as a protection measure (Davis et al. 1986). This hypothesis is supported by a study showing that Suncus murinus (house musk shrew), a species capable of vomiting, will not avoid a flavour that has been paired with drugs that are capable of producing a conditioned place preference, in fact they will develop a taste preference for that flavour instead (Parker et al. 2002). Therefore, concluding that development of CTA for a drug-paired flavour indicates that the avoidance is caused by aversive properties of the drug may not be accurate. Indeed, assessing the effects of cocaine in a traditional TR test, a paradigm that does not require the animal to approach the flavour, we see that aversive behaviours do not develop. Specifically, Parker (1993) found that when administering varying doses of sc cocaine immediately following a two-minute intraoral infusion of sucrose in the taste reactivity test, rats did not develop taste aversion (e.g., gaping) to the flavour, however they showed a decrease in ingestive responses during the TR test, as well as a taste avoidance when rats had the opportunity to ingest the sucrose in a bottle test.
Although pairing a 2-minute intraoral infusion of sucrose with a subsequent injection of cocaine does not result in the development of aversive behaviours (Parker 1993), pairing saccharin with delayed access to cocaine does result in an aversion to the saccharin. Wheeler et al. (2008) demonstrated that multiple brief (3.5 sec) exposures to saccharin once a minute over 30 minutes paired with delayed access to cocaine in a self-administration task, ultimately resulted in rats displaying conditioned gaping to the saccharin solution over the 30 min waiting period. Wheeler et al. (2008) hypothesised that the initial “drug waiting” period results in a conditioned aversion to the flavour paired with delayed access to cocaine. They state that “the cocaine-paired taste served as a predictive cue of cocaine’s impending availability and precipitated the expression of a cocaine aversive state in learned anticipation of the future opportunity to self-administer cocaine” (Wheeler et al. 2008). They suggested that the “learned cocaine aversive state” represented a state of cocaine withdrawal; a cocaine-need state. In addition to the finding that rats gape to a flavour that has been paired with delayed access to cocaine, they found that the amount of gaping predicted the amount of cocaine rats subsequently self-administered. More specifically, rats who showed high aversive taste reactivity had a shorter latency to first-press and larger drug loading (amount of lever presses for first 5 minutes) during the cocaine self-administration period. This finding supports previous research showing that negative affect drives drug seeking (Baker et al. 2004; Koob and LeMoal. 1997).

The learned cocaine aversion observed in the Wheeler et al. (2008) self-administration study was subsequently replicated with delayed delivery of ip cocaine and was therefore not a unique effect of self-administration recruiting the mesolimbic dopamine (DA) system (eg.
The nucleus accumbens (NAc) is a brain region associated with this system and it plays an important role in modulating motivated behaviours (Wise 2004). It has also been shown to be involved in the devaluation of natural reward, a phenomenon that is common in individuals suffering from cocaine addiction. Using electrophysiological measures, Wheeler et al. (2008) found that rats who are administered saccharin that had not been paired with delayed access to cocaine showed a reduced firing rate in the NAc, whereas administration of saccharin that signaled delayed access to cocaine produced an increased firing rate of NAc neurons. These results were similar to previous findings that administration of a palatable solution produces inhibitory responses, while administration of an aversive solution produces predominantly excitatory responses in the NAc (Roitman et al. 2005). In accordance with this finding, using fast-scan voltammetry, a technique that allows direct neurotransmitter readings in a behaving animal, Wheeler et al. (2011) observed decreased mesolimbic DA concentrations to the cocaine-predictive cue that signaled delayed cocaine availability, but this switched to elevated mesolimbic DA concentrations to taste cues signaling imminent cocaine delivery in a self-administration session. These findings suggest that a taste cue signaling delayed access to cocaine results in drastically different NAc firing and subsequent mesolimbic DA concentrations than a cue predicting immediate cocaine availability, that may be representative of a negative affective state in the rats (Wheeler et al. 2011).

Aversive Properties of Cocaine

The effect of delayed access to reward producing conditioned aversion to saccharin solution may be specific to the affective properties of cocaine, itself, rather than to the
“anticipation of impending reward” as suggested by Wheeler et al. (2008; 2011). Unlike heroin, amphetamine or food reward, Ettenberg (2004) has suggested that while the immediate effects of cocaine are rewarding, it actually becomes aversive soon after these positive effects. Ettenberg et al. (1999) showed that in a conditioned place preference (CPP) paradigm, a context that has been paired with immediate cocaine delivery produces a preference for that place, however if a short delay (15 min) is introduced in the pairing of cocaine and a context, rats will develop a conditioned place aversion and spend less time on that side of the box. This supports the hypothesis that, while cocaine is rewarding, it becomes aversive shortly after the initial experience. In another experiment, Ettenberg (2004) assessed the effects produced by cocaine reward in a runway task. The runway task uses a straight alley that the rat must run down in order to reach a goal box where it receives a reward, and it is meant to determine the motivational state of the animal. It is observed that animals will run faster down the alley to the goal box when rewarded with food, water, or certain rewarding drugs, such as heroin, and amphetamine (Chausmer and Ettenberg 1997; Ettenberg and Camp 1986; Ettenberg 1990; McFarland & Ettenberg, 1995). However, when Ettenberg (2004) employed this task so that rats who entered the goal box were intravenously (iv) infused with cocaine, they found different approach behaviours than are typically seen with reinforcing stimuli. Rats would display similar quick start latencies when compared to rewarding stimuli, however they exhibit a distinct “stop and retreat” behaviour that occurs multiple times before entering the goal box. This behaviour pattern is similar to that of a rat that received a food reward coupled with foot shock upon entering the goal box. This study further demonstrates that cocaine produces negative effects in addition to its rewarding properties. Therefore, the generality of the potential of delayed access
to cocaine to produce conditioned aversive response needs to be evaluated with other rewarding drugs.

Present Study

It is important to find a model that can directly measure the negative affective state caused by cocaine-associated cues in order to further our understanding of how this aversive state contributes to drug craving and seeking. Most models of drug seeking do not consider the emotional or affective component of drug use, or how drug-associated cues may contribute to a negative affective state (Wheeler et al. 2008). However, research provides strong evidence that conditioned gaping to saccharin paired with impending cocaine is a behaviour that predicts cocaine self-administration, which indicates that this model can be a measure of both the emotional impact of cocaine-related cues and how these cues lead to motivated drug seeking behaviour. Specifically, it is suggested that this conditioned gaping is an index of a negative affective state that can predict future cocaine self-administration (Wheeler et al. 2008; Wheeler et al. 2011). The experiments described in this thesis further examined the negative affective component of this model, particularly how saccharin is able to act as a cue for delayed access to cocaine and as such is able to elicit an aversive state, as evidenced by conditioned gaping.

Firstly, the parameters by which cocaine could elicit the aversive conditioned gaping reaction were examined. In a series of experiments, saccharin was paired with either immediate or delayed cocaine administration. The immediate procedure consisted of a 2-min intraoral infusion of saccharin typical of traditional TR experiments, and the delayed procedures administered saccharin intermittently for either 10-min or 30-min before cocaine administration.
Two routes of cocaine administration were tested – either sc or ip – because of differing effects of these routes on behaviour (Mayer and Parker 1993; Ferrari et al. 1991). The immediate and 30-min delay procedure were also repeated with lithium chloride (LiCl) for comparison with cocaine administration to that of a known aversive drug. Since a 10-min delay was sufficient to produce an aversion to saccharin paired with cocaine, it was also used to determine whether a rewarding drug other than cocaine, specifically morphine, was able to elicit a conditioned aversion to saccharin. Because cocaine withdrawal is associated with anxiety (Kupferschmidt et al. 2012), the ability of delayed cocaine-paired saccharin to elicit anxiety-like behaviour in the Light-Dark (LD) Emergence test was also examined. In another experiment saccharin was administered for a continuous 2 minutes, followed by a 30-min delay to cocaine administration. Finally, potential treatments to alleviate the conditioned aversive state produced by saccharin paired with a 10-min delay to cocaine was examined. Specifically, the ability of ondansetron – a 5-HT3 receptor antagonist commonly prescribed for the management of nausea and vomiting – to interfere with conditioned gaping to saccharin paired with delayed cocaine was determined. The dopamine D1 receptor antagonist SCH 23390 was also tested because of the importance of these receptors in the development of drug dependence. Finally, a newly discovered endocannabinoid-like compound, oleoyl glycine (OGLy), that interferes with LiCl induced nausea and naloxone-precipitated morphine withdrawal (Rock et al. submitted) was evaluated for its potential to interfere with conditioned gaping to saccharin paired with delayed cocaine administration.
Conditioned aversive responses produced by delayed, but not immediate, exposure to cocaine in male Sprague-Dawley rats

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Rationale To determine the conditions under which tastes paired with delayed access to experimenter-delivered cocaine and morphine elicit a conditionally aversive affective state.

Objectives and methods The potential of saccharin paired with immediate access to cocaine (5, 10, 20 mg/kg, sc and ip) and delayed (30 and 10 min) access to cocaine (20 mg/kg, sc and ip) and morphine (10 mg/kg, sc) to elicit a pattern of aversive responding in the taste reactivity test (Grill and Norgren 1978a) was evaluated. Cocaine-induced aversions were compared with those produced by a moderate dose of LiCl (50 mg/kg). Finally, as an independent measure of cocaine withdrawal, the potential of exposure to saccharin paired with delayed access to cocaine to produce anxiogenic-like responding in the Light-Dark Emersion test was evaluated.

Results Immediate access to cocaine did not produce conditioned aversion at any dose. Delayed (30 or 10 min) access to sc cocaine (20 mg/kg) produced robust conditioned aversion and delayed access to ip cocaine (20 mg/kg; 30-min) and to sc morphine (10 mg/kg; 10-min) produced weaker conditioned aversion. Yawning emerged as a potential withdrawal response in rats conditioned with delayed (30 min) access to 20 mg/kg, sc, cocaine. Contextual cues did not produce conditioned aversion when paired with delayed access to sc cocaine (20 mg/kg). Finally, exposure to saccharin paired with delayed access to cocaine produced anxiogenic-like responding in the Light-Dark Emersion test.

Conclusion Our results support the contention that a conditioned aversive state develops when a taste cue comes to predict the delayed availability of drugs of abuse.

Keywords: cocaine, negative affect, withdrawal, taste reactivity, aversion, morphine, LiCl, rat
Introduction

Cocaine abuse is accompanied by the emergence of negative affect such as dysphoria, irritability and anhedonia. Indeed, this negative affect plays a prominent role in craving and relapse in animal models (e.g. Koob and LeMoal 1997; Solomon and Corbit 1974; Wheeler et al. 2008); the greater these negative feelings, the greater the subjective euphoric effect of subsequent cocaine administration in humans (Newton et al. 2003; Sofuoglu et al. 2003). The negative affective state can become associated with cues that trigger craving and relapse, and, consequently, the subsequent euphoric effect of the cocaine administration. Recently, using the taste reactivity test (Grill and Norgren 1978a), Wheeler et al. (2008) directly measured this negative affect in rats by demonstrating that a taste cue paired with delayed access to cocaine elicits a conditioned aversive state that is quantifiable and predicts greater subsequent cocaine intake.

The taste reactivity (TR) test (Grill and Norgren 1978) is a direct measure of the hedonic valence of the taste stimulus (Berridge 2000). These stereotyped oromotor responses to palatable (sucrose) and unpalatable (quinine) tastes reflect not only innate reactions, but also conditioned changes in affect that are dissociable from simple ingestive behavior. Rats avoid intake of a taste cue that has been paired with a drug that produces nausea such as lithium chloride (LiCl). They also display the aversive reactions of gaping, chin rubbing and paw treading to such a conditioned aversive taste (Grill & Norgren, 1978b). Rats, which cannot vomit, also avoid intake of a taste cue that has been paired with a self-administered drug of abuse, such as cocaine (e.g. Parker 1993); and, indeed, greater avoidance of the taste cue is associated with greater cocaine self-administration (Grigson and Twining 2002). On the other hand, when a taste cue is paired
immediately with a drug of abuse, such as cocaine and morphine, rats do not subsequently respond to that taste with the aversive reactions of gaping, chin rubbing and paw treading, although they suppress ingestion (tongue protrusions) and allow the taste to passively drip from their mouth (avoidance response) (Parker 1993; Parker 1995).

Wheeler et al. (2008) reported that following several pairings of multiple exposures to a saccharin cue with the delayed opportunity to self-administer cocaine, rats eventually display conditioned gaping reactions during the waiting period. In fact, the greater the cocaine intake in the self-administration period, the greater the conditioned gaping to the saccharin during the delay. The development of gaping reactions elicited by a taste that predicted delayed access to cocaine was subsequently replicated with delayed delivery of intraperitoneal (ip) cocaine, and was therefore not a unique effect of the potential of self-administered cocaine to recruit the mesolimbic dopamine system (e.g. Hemby et al. 1997; Stuber et al. 2005; Kippin et al. 2006). Wheeler et al. (2008) hypothesized that, “the cocaine-paired taste served as a predictive cue of cocaine’s impending availability and precipitated the expression of a cocaine aversive state in learned anticipation of the future opportunity to self-administer cocaine.” They suggested that the “learned cocaine aversive state” represented a state of cocaine withdrawal (a cocaine-need state). That is, the rats developed a compensatory conditioned response (Siegel & Ramos, 2002) or a conditioned “b” state (Solomon and Corbit, 1974) that became associated with the taste. Indeed, McDonald et al. (1997) reported that taste cues associated with naloxone-precipitated opiate withdrawal elicited conditioned gaping reactions in rats.

What is unique about the Wheeler et al. (2008, 2011) procedure is that the taste cue was intraorally delivered at 3.5-s intervals across a 30-45 min period prior to cocaine availability in a
self-administration paradigm constituting a “drug waiting” period that may promote an association between the taste and the negative affective state as revealed by conditioned gaping. Parker (1993, 1995) administered cocaine by subcutaneous (sc) injection immediately following a 2-min intraoral infusion of saccharin and found no evidence of gaping to the cocaine paired saccharin solution even after five conditioning trials (spaced 72 h apart). The present series of experiments first replicated the experiments of Wheeler et al. (2008) and Parker (1993, 1995), with experimenter-delivered ip and sc cocaine and compared these results with a moderate dose of LiCl (50 mg/kg, ip) using both procedures. Cocaine was injected both sc and ip, because when administered sc (relative to ip), it is more effective in producing conditioned taste avoidance (Mayer and Parker 1993; Ferrari et al. 1991), but when cocaine is administered ip (relative to sc) it is more rewarding in a place preference paradigm (Mayer and Parker 1993; Tzschentke 1998). If the potential of a conditioned cocaine waiting period to produce gaping relies on the rewarding effects of cocaine, then ip administered cocaine may be more effective in producing gaping than sc cocaine, even though sc cocaine is more likely to produce a taste avoidance response. Subsequent experiments evaluated the potential of a contextual stimulus in the absence of a taste to elicit aversive reactions followed by a delayed (30 min) injection of cocaine. We also evaluated the potential of repeated exposure to saccharin over a 10-min period of delayed access to both cocaine (20 mg/kg, sc) and another rewarding drug, morphine (10 mg/kg, sc), to produce aversive responding, as a test of generality across drugs of abuse. Finally, since cocaine withdrawal is characterized by a high state of anxiety-like responding (Kupferschmidt et al, 2012), we evaluated the potential of repeated exposure to saccharin over a 30 min period prior to
cocaine (20 mg/kg, sc) to produce anxiogenic-like responding in the Light-Dark (LD) Emergence test.

**Methods**

**Subjects**

The subjects were 160 male Sprague-Dawley rats (Charles River, QC). Rats were between 250 and 300g in weight on the first conditioning trial. Rats were individually housed in a colony room on a 24-h light-dark cycle (7am, lights off; 7pm, lights on) such that behavioral testing was conducted during the dark phase of the light cycle. Rats were maintained on ad libitum rat chow and water, except when indicated otherwise.

**Surgery**

Rats were surgically implanted with an intraoral (IO) cannula 1 week prior to conditioning. Implantation of the cannula was done while rats were under isoflurane anaesthesia according to the procedures previously described (Limebeer et al. 2010). Rats underwent 3 days of post-surgical monitoring beginning the day following surgery.

**Drugs**

Cocaine was mixed at a concentration of 1.5 mg/ml in solution with saline (SAL) for subcutaneous injection in order to prevent skin necrosis. As well, the injection site was changed on a daily basis. There were no instances of necrosis with these precautions. Injection volumes were dependent on body weight and dose. The 5 mg/kg dose was administered at 3.3 ml/kg, 10
mg/kg at 6.7 ml/kg, and 20 mg/kg at 13.3 ml/kg. For intraperitoneal (ip) injections, cocaine was mixed at a concentration of 5, 10, and 20 mg/ml. Volume of injection was dependent on body weight and was prepared at 1ml/kg. In order to equate the volume of morphine and cocaine, in experiment 4, morphine was also prepared at a concentration of 1.5 ml/ml and administered at a volume of 6.7 ml/kg sc. LiCl was prepared as a 0.15 M solution in sterile water and administered at a volume of 8 ml/kg ip (50 mg/kg).

**Apparatus**

For taste reactivity measures, the rats were placed in a clear Plexiglas box (22.5 x 26 x 20 cm) with an opaque lid, sitting on top of a clear glass-topped table. A mirror was located under the chamber at a 45° angle in order to allow viewing of the rat ventral surface. The chamber was located in a dark room next to a 25-W light source. There was a video camera (Sony DCR-HC48, Henry’s Cameras, Waterloo, ON, Canada) placed in front of the mirror to allow the trials to be recorded and scored at a later time. Trials were scored by an observer blind to the experimental groups using “The Observer” (Noldus Information Technology Inc., Leesburg, VA, USA).

For Experiment 3, the distinctive context was an opaque black Plexiglas box (22.5 x 26 x 20 cm) with an opaque lid, sitting on top of a clear glass-topped table. Closed circuit cameras located beneath each chamber pointed toward the rat to allow later scoring for gaping and the activity of the rat was measured using the Ethovision software program (Noldus, Inc., NL) to measure distance (cm) traveled.
For Experiment 5, the LD Emergence apparatus has been previously described by Rock et al. (2017). A video camera was mounted over the top of the light-dark box and the videotapes were analyzed by Ethovision software (Noldus Information Technology, Leesburg, VA, USA) for the duration of time spent in the light box during the 5-min test.

Taste Reactivity Measures

The literature on taste reactivity is somewhat inconsistent in the definition of aversive responses, especially when the responses are combined into a total aversive response score; therefore, we have chosen to individually measure each behavior described by Grill and Norgren (1978a) separately. Given that the definition of each of these behaviors is consistently applied across laboratories, such an analysis gives sufficient information to facilitate replication of results. Although Grill and Norgren (1978a, b) initially identified gaping, chin rubbing, paw treading, head shakes, face washes, forelimb flailing and increased locomotion as aversive responses (which are often summed), a subsequent factor analysis (Parker 1995) indicated that taste reactivity responses tend to cluster into aversive responses, motoric responses and ingestion/non-ingestion related responses. Each of the behaviors measured is described in Table 1. Aversive behaviors include gaping, chin rubbing and paw treading. Motoric responding behaviors include active locomotion, rearing, face washing, head shakes and forelimb flails. Ingestion/non-ingestion related responses included the positive hedonic response of tongue protrusions, the avoidance-like response of passive dripping, and avoidance of the taste (conditioned taste avoidance, CTA). As well, since the taste reactivity test also provides the opportunity for detecting other orofacial reactions, we have included the response of yawning,
which has been reported in animals (Nakamura-Palacios et al. 2002; Jaw et al. 1993; Schnur et al. 1992) and humans (Bickel et al. 1988) undergoing acute withdrawal from several drugs of abuse.

Procedure

Experiment 1: immediate pairing

Experiment 1a: immediate sc cocaine All rats were adapted to the taste reactivity chamber 3 days prior to the first conditioning trial. During adaptation, the rat was placed in the taste reactivity chamber and its cannula was attached to an infusion pump (Model KDS100; KD Scientific, Holliston, MA, USA) for fluid delivery. For adaptation, reverse osmosis water was infused into the intraoral cannula at a rate of 1ml/min for a total of 2 min.

Three days following adaptation, the first conditioning trial began. On each of four conditioning trial/testing trials, the rat was placed in the taste reactivity chamber, and its cannula was attached to the pump for the infusion of 0.1% saccharin at a rate of 1 ml/min for 2 min. The timer started when the rat made an orofacial response and stopped at 2 min. The trial was recorded for scoring at a later time. At the end of the 2-min infusion session on trials 1-3, the rat was removed from the chamber and given an immediate sc injection of saline ($n = 8$) or cocaine at a dose of 5 mg/kg ($n = 7$), 10 mg/kg ($n = 7$) or 20 mg/kg ($n = 7$). Following the injection, the rat was placed back in its home cage. The TR test was conducted on the fourth day in the same manner as the conditioning trials but with no subsequent injection.
At 16:00 on the day after the testing, the water bottles were removed from each rat’s cage. At 9:00 on the following day, rats were given 0.1% saccharin solution in graduated tubes and the amounts consumed at 30, 120, 360 and 240 min were measured as a measure of CTA.

*Experiment 1b: immediate ip cocaine* All adaptation and conditioning procedures were conducted as outlined in experiment 1a. During the three conditioning trials, following the 2-min infusion of saccharin solution, rats were injected ip with saline ($n = 8$), or 5 mg/kg ($n = 7$), 10 mg/kg ($n = 7$) or 20 mg/kg ($n = 6$) cocaine. The drug-free test trial was conducted on the fourth day and the CTA test on the following day as outlined in experiment 1a.

*Experiment 1c: immediate ip LiCl* All adaptation and conditioning procedures were conducted as outlined in experiment 1a. During the three conditioning trials, following the 2-min infusion of saccharin, the rats were injected ip with saline ($n = 8$) or 50 mg/kg (8 ml/kg 0.15 M) LiCl ($n = 8$). The drug free test trial was conducted on the fourth day and CTA test on the following day as outlined in experiment 1a.

*Experiment 2: delayed (30-min) saccharin pairing with cocaine (20 mg/kg, sc and ip) and LiCl (50 mg/kg, ip)*

All rats were adapted to the taste reactivity procedure 3 days prior to the first conditioning trial. During adaptation, the rat was placed in the taste reactivity chamber and its cannula was attached to an infusion pump (Model KDS100; KD Scientific, Holliston, MA, USA) for fluid delivery. During the adaptation trial, each rat was intraorally infused with reverse osmosis water on each of 30 trials for 10 s/trial at the rate of 1.2 ml/min every min over 30 min.
Three days following adaptation, the first conditioning trial began. On each of four conditioning trial/testing trials, the rat was placed in the taste reactivity chamber and its cannula was attached to the pump for the infusion of 0.1% saccharin and infused according to the same schedule as water during adaptation. After the 30th infusion, on conditioning trials 1-4, the rat was removed from the chamber and given an immediate injection of saline ($n = 6$ half sc/half ip), 20 mg/kg, sc, cocaine ($n = 6$), 20 mg/kg, ip, cocaine ($n = 6$) or 50 mg/kg (8 ml/kg 0.15 M) LiCl ($n = 6$). Following the injection, the rat was placed back in its home cage. The TR test trial occurred 24 h after the final conditioning trial. The rats were given a CTA test 24 h after the test trial as in experiment 1.

**Experiment 3: delayed (30-min) context pairing with cocaine**

In experiment 2, rats were exposed to saccharin in the TR chamber, which is distinct from their home cage. Therefore, to ensure that the aversive taste reactions were elicited by the taste rather than by the conditioning context, experiment 3 evaluated the potential of a distinctive context to elicit aversive reactions following repeated pairings with cocaine upon removal from the context 30 min later. Limebeer et al. (2008) have shown that rats will learn to gape to a distinctive context (even in the absence of a taste) if they experience LiCl-induced nausea while in that context.

The rats received four conditioning trials, one per day on consecutive days, followed by a drug free test trial 24 h later. On each trial, they were placed in the distinctive context for 30 min. They were then immediately removed from the chamber and given an injection of either saline ($n = 8$) or 20 mg/kg sc cocaine ($n = 8$). On the test trial, the behavior of the rat was recorded with
closed circuit cameras located beneath each chamber and the image was sent to Ethovision software to measure distance (cm) traveled, which was analyzed in 5-min intervals across the 30-min test. As well, the behaviors of gaping, chin rubbing, paw treading, and yawning were scored from the videotapes.

**Experiment 4: delayed (10 min) saccharin pairing with cocaine or morphine**

The procedures of experiment 4 were similar to those of experiment 2 except that the rats were injected with sc saline (n = 8), 20 mg/kg sc cocaine (n = 7), or 10 mg/kg sc morphine (n = 8) 10 min following repeated 10-s (every minute) saccharin exposures in the TR test. This provides a control for amount of saccharin exposure on the development of conditioned aversive responses because the total amount of saccharin infused was 2 ml (as in experiment 1) across the 10-min, trials rather than 6 ml (as in experiment 2) across the 30-min trials. As well in the CTA test, there was no 30-min measure taken.

**Experiment 5: assessment of anxiogenic-like responding following exposure to saccharin previously paired with delayed access to cocaine**

A total of 14 rats were conditioned as in experiment 2 such that half (n = 7) received three daily conditioning trials with repeated saccharin infusions (10 s) every min for 30 min prior to cocaine (sc) and the other half (n = 7) prior to saline (sc). On the following day, the rats received saccharin as during conditioning, but after 30 min were placed in the dark corner of the LD emersion box facing away from the opening between the two chambers and the movement of the rat was tracked during the 5-min test. An additional two groups of rats received three daily
unpaired home cage injections of cocaine \( (n = 8) \) or saline \( (n = 8) \) and on the next day were given the same LD test described above, to evaluate the effect of cocaine exposure on anxiogenic-like behavior 24 h later.

*Data Analysis*

The primary measures to be compared across experiments included the aversive reactions of gaping, chin rubbing, paw treading, the positive hedonic reaction of tongue protrusions, the avoidance behaviours of passive drips, and CTA. In experiments 1 and 4, the frequency or duration of each TR behavior above on each conditioning/testing trial for each group was entered into a mixed-factors analysis of variance (ANOVA). As well, the frequency or duration of the motoric TR behaviors displayed on the final TR test trial were analyzed as single factor ANOVAs. To evaluate CTA in Experiments 1, 2 and 4, the mean cumulative amount (ml) saccharin solution consumed at each interval of testing for each group was entered into a mixed factors ANOVA. In Experiment 2, the TR test trial data for the entire 30-min test trial was evaluated at each 5-min interval, which included summed reactions during and between saccharin infusions. The 5-min interval scores for each behavior on the test trial for each group were entered into a mixed-factors ANOVA. The total frequency or duration of each motoric TR behavior displayed during the 30-min test trial was analyzed as single factor ANOVA. In experiment 3, the number of gapes and the distance traveled per 5-min interval for each group were entered into a mixed-factors ANOVA. In experiment 5, the TR behaviors of gaping, chin rubbing, paw treading, yawning, passive drips and tongue protrusions and the time (s) spent in the lit box by the rats conditioned with delayed cocaine and the rats conditioned with delayed
saline were compared by \( t \) tests. As well the time (s) spent in the lit box by the home cage cocaine and home cage saline groups were compared by a \( t \) test. Significance was defined as \( p < 0.05 \).

Results

Experiment 1: immediate conditioning

When administered immediately following 2-min exposures to saccharin, cocaine (sc or ip) did not produce any of the aversive behaviors of gaping, chin rubbing, or paw treading across the four conditioning/testing trials at any dose. In contrast, the moderate dose of LiCl produced conditioned gaping, chin rubbing, and paw treading across trials. As previously reported (Parker 1993), and as is also seen with LiCl, cocaine delivered sc suppressed hedonic tongue protrusions and produced CTA across all doses and at 20 mg/kg increased passive dripping. When cocaine was delivered ip, only 20 mg/kg suppressed tongue protrusions, increased passive dripping, and produced a CTA. Among the motoric responses, the groups conditioned with 20 mg/kg, sc, cocaine showed more head shakes on the test trial than those conditioned with saline. In addition, LiCl conditioned rats showed less face washing (often considered to be an aversive reaction) than saline conditioned rats, but they did not differ in any other motoric behavior across trials.

Experiment 1a: immediate sc cocaine

Figure 1 presents the mean (± SEM) number or duration of gaping, chin rubbing, paw treading, tongue protrusions, and passive drips, as well the CTA measured for the groups conditioned with sc cocaine. Yawning is not depicted in any table or figure of experiment 1a–c because the rats did not display yawning. The 4x4 mixed-factors
ANOVA for the behaviors of gaping, chin rubbing, and paw treading revealed no significant effects.

The 4x4 mixed-factors ANOVA for tongue protrusions revealed a significant group effect, \(F(3, 25) = 4.6, p < 0.01\), and an effect of trial, \(F(3, 75) = 6.0, p < 0.01\). An analysis of each trial revealed that the groups significantly differed only on trial 3, \(F(3,28) = 5.7, p = 0.004\) and trial 4 \(F(3, 28) = 12.5, p < 0.001\); subsequent Bonferroni tests revealed that all sc cocaine doses produced suppressed tongue protrusions on trials 3 and 4 relative to group saline (\(p\)'s < 0.05).

For the behavior of passive drips, the 4x4 mixed-factors ANOVA revealed significant effects of group, \(F(3, 25) = 6.9, p < 0.001\), and a group x trial interaction, \(F(9, 75) = 2.4, p = 0.019\). The groups differed on conditioning trial 3, \(F(3, 25) = 6.4, p = 0.002\), and on the test trial, \(F(3, 25) = 5.4, p = 0.005\); subsequent Bonferroni tests revealed that on each of these trials, group 20 mg/kg sc cocaine displayed more passive drips than group saline \((p < 0.01)\). Finally, the ANOVA for the CTA revealed significant effects of time \(F(3, 75) = 185.3, p < 0.001\), group x time \(F(9, 75) = 6.8, p < 0.001\), and a main effect of group \(F(3, 25) = 10.6, p < 0.001\). Subsequent Bonferroni post hoc comparisons of the group effect revealed that all three doses of cocaine suppressed saccharin intake across the 6 h CTA test relative to group saline \((p\)'s < 0.05) and the cocaine doses did not differ from one another at any interval of testing.

Of the motoric behaviors assessed depicted in Table 1 (active locomotion (s), rearing (s), face washing (s), head shakes (f), forelimb flails (f)), the single-factor ANOVA for experiment 1a revealed only a significant effect for head shakes, \(F(3, 25) = 6.4, p < 0.01\), with the group
conditioned with 20 mg/kg, sc, cocaine showing more \((p < 0.01)\) head shakes than group saline or 10 mg/kg, sc cocaine (data not depicted).

**Experiment 1b: immediate ip cocaine** Figure 2 presents the mean (+SEM) number or duration of the primary TR reactions measured in experiment 1b with ip cocaine. The 4x4 ANOVA for the behaviors of gaping, chin rubbing, and paw treading revealed no significant effects.

The 4x4 mixed-factors ANOVA for the number of tongue protrusions revealed only a significant main effect of trials, \(F(3, 72) = 3.0, p = 0.037\). To evaluate each trial separately, single-factor ANOVAs revealed that groups only differed on trial 4, \(F(3, 27) = 4.3, p < 0.015\); Bonferroni post hoc comparison tests revealed that group 20 mg/kg, ip, cocaine displayed significantly fewer tongue protrusions on the final test trial than groups saline or 5 mg/kg cocaine \((p's < 0.05)\).

The 4 x 4 mixed-factors ANOVA for the number of passive drips revealed a significant effect of group, \(F(3, 24) = 6.0, p < 0.001\); trial, \(F(3, 24) = 5.7, p = 0.004\); and a group x trial interaction, \(F(9, 72) = 6.0, p < 0.001\). To evaluate the interaction, single-factor ANOVAs for each trial revealed a group effect only on trials 3 and 4 \((p's < 0.01)\); subsequent Bonferroni post hoc comparison tests revealed that group 20 mg/kg ip cocaine displayed more passive drips than group saline or 5 mg/kg ip cocaine on each of these trials \((p's < 0.025)\). Finally, the ANOVA for the CTA revealed significant main effects of group, \(F(3, 24) = 7.3, p < 0.001\); time, \(F(3, 72) = 157.1, p < 0.001\); and a group x time interaction, \(F(9, 72) = 3.2, p < 0.01\). Bonferroni post hoc comparisons of the group effect revealed that a dose of 20 mg/kg, ip, cocaine suppressed saccharin intake overall relative to saline and 5 mg/kg, ip, cocaine \((p < 0.05)\). Bonferroni tests for
each interval also revealed that at 30 min and at 120 min only, group 10 mg/kg, ip, cocaine drank less than group saline \((p < 0.05)\), but this was overcome with further exposure to saccharin.

The single-factor ANOVA for the motoric responses assessed in experiment 1b on the final test trial revealed no significant effects (data not depicted).

**Experiment 1c: immediate ip LiCl** Figure 3 presents the mean (± SEM) number or duration of the primary TR behaviors displayed by the groups in experiment 1c. For the behaviors of gaping, chin rubbing, and paw treading, the 2 x 4 ANOVAs revealed a significant main effect of group (gapes, \(F(2, 21) = 29.2, p < 0.001\); chin rubs, \(F(2, 21) = 20.5, p < 0.001\); paw treads, \(F(2, 21) = 6.7, p = 0.006\)), trials (gapes, \(F(3, 63) = 32.7, p < 0.001\); chin rubs, \(F(3, 63) = 16.7, p < 0.001\); paw treads, \(F(3, 63) = 14.1, p < 0.001\)), and a group x trials interaction (gapes, \(F(3, 63) = 10.1, p < 0.001\); chin rubs, \(F(3, 63) = 224.9, p < 0.001\); paw treads, \(F(3, 63) = 5.2, p < 0.001\)). Subsequent independent \(t\) tests revealed that group LiCl displayed significantly more gaping and chin rubbing on trials 2–4 \((p's < 0.001)\) and displayed more paw treading on trials 3–4 \((p's < 0.05)\) than group saline.

The 2 x 4 ANOVA for the positive hedonic reaction of tongue protrusions revealed significant main effects of group, \(F(2, 21) = 16.9, p < 0.001\); trials, \(F(3, 63) = 6.7, p = 0.001\); and a group by trials interaction, \(F(3, 63) = 7.0, p < 0.001\); group LiCl displayed fewer tongue protrusions than group saline on trials 2–4 \((p's < 0.01)\).

For the reaction of passive dripping, the ANOVA revealed significant main effects of group, \(F(2, 21) = 17.7, p < 0.001\); trials, \(F(3, 63) = 16.7, p < 0.001\); and a group x trials interaction, \(F(3, 63) = 84.0, p = 0.002\); group LiCl displayed enhanced passive dripping on trials 2–4 \((p's < 0.05)\). Finally for the CTA, depicted in the bottom left corner of Fig. 2, the 2 x 4
mixed-factors ANOVA revealed significant main effects of group, $F(1, 14) = 62.7, p < 0.001$; interval, $F(1, 14) = 83.4, p < 0.001$; and a group x interval interaction, $F(1, 14) = 70.2, p < 0.001$; group LiCl displayed suppressed intake in the CTA test at all intervals relative to group saline ($p < 0.01$). The independent $t$ tests for the motoric responses on the TR trial in experiment 1c revealed only that group LiCl displayed significantly less face washing than group saline, $t(14) = 3.3; p < 0.01$; no other behaviors differed between the groups (data not depicted).

Experiment 2: delayed (30 min) saccharin pairing with cocaine (20 mg/kg, sc and ip) and LiCl (50 mg/kg, ip)

The primary TR aversive behaviors (gaping, chin rubbing, paw treading) as well as the potential withdrawal-related measure of yawning, the positive hedonic reactions of tongue protrusions, and the avoidance-like behavior of passive drips display by the various groups on the final TR test trial across 5-min intervals are depicted in the upper section of Fig. 4. As well in the lower right-hand corner of Fig. 4, the CTA measure across intervals is depicted. When saccharin predicted the delayed delivery of sc cocaine or LiCl, rats displayed conditioned gaping and chin rubbing which was more pronounced during the early intervals of testing. However, only LiCl conditioned rats displayed the aversive behavior of paw treading. Only rats conditioned with sc cocaine displayed the withdrawal-like response of yawning. Rats conditioned with delayed access to cocaine (both ip and sc) or LiCl displayed suppressed tongue protrusions; however, only cocaine sc and LiCl produced enhanced passive dripping. Finally, all three drug groups showed suppressed consumption relative to group saline in the CTA test and did not differ from one another overall. Both sc cocaine and LiCl suppressed face washing and sc
cocaine produced more head shaking than LiCl on the final test trial. The results of the analysis of each behavior is described below.

**Aversive TR responses** For the gaping measure, the 4 x 6 mixed-factors ANOVA of the number of gapes per 5-min interval for the various groups revealed a significant effect of group, $F(3, 20) = 4.8, p = 0.011$. Bonferroni pairwise comparison tests for the main effect across time revealed that both cocaine sc and LiCl produced significantly more gaping than saline ($p$’s < 0.05), but not cocaine ip. Because we predicted that ip cocaine would produce conditioned gaping under conditions of delay (Wheeler et al. 2008), a less conservative least significant difference (LSD) comparison test revealed that ip cocaine also enhanced gaping overall ($p < 0.05$). The analysis also revealed significant effects of time, $F(5, 100) = 7.6, p < 0.001$ and a group x time interaction, $F(15, 100) = 3.9, p < 0.001$. Bonferroni post hoc comparison tests revealed that during the first and second 5-min intervals, both groups cocaine sc and LiCl displayed more gaping than group saline ($p$’s < 0.05). Group cocaine ip did not differ from any other group at any interval. For chin rubbing, the ANOVA revealed an effect of time, $F(5, 100) = 45.4, p < 0.001$, and a group x time interaction, $F(15, 100) = 2.7, p = 0.002$. Bonferroni tests revealed that during the first 5-min interval, both LiCl and sc cocaine produced more chin rubbing than either ip cocaine or saline ($p$’s < 0.05). For paw treading, the ANOVA revealed a main effect of group, $F(3, 20) = 5.4, p = 0.007$, with subsequent Bonferroni post hoc tests on the main effect indicating that group LiCl displayed more paw treading than any other group ($p$’s < 0.05). The effect of time, $F(5, 100) = 3.1, p < 0.05$, and the group x time interaction, $F(15, 100) = 2.2, p = 0.011$, were also significant, with Bonferroni tests revealing that LiCl produced more paw treading than all treatments during all intervals but the fourth.
Withdrawal-like response The withdrawal-like response (B) of yawns are depicted in the upper right-hand corner of Fig. 4. The ANOVA revealed only a significant main effect of group, \( F(3, 20) = 9.5, p < 0.001 \); subsequent Bonferroni tests revealed that cocaine sc produced significantly more yawns over the 30-min test than any other treatment (\( p \)'s < 0.01). This may represent a conditioned withdrawal response to cocaine that is also seen with opiates (e.g., Schnur et al. 1992; Bickel et al. 1988).

Tongue protrusions, passive drips and CTA For tongue protrusions, the ANOVA revealed a significant main effect of group, \( F(3, 20) = 33.5, p < 0.001 \), and a group x time interaction, \( F(15) = 3.2, p = 0.015 \); subsequent Bonferroni tests revealed that during each interval, group saline showed more tongue protrusions than all other groups (\( p \)'s < 0.01), which did not differ from one another at any interval. For passive drips, the ANOVA revealed a significant main effect of group, \( F(3, 20) = 7.3, p = 0.002 \); time, \( F(5, 100) = 3.7, p = 0.004 \); and a group x time interaction, \( F(15, 100) = 2.0, p < 0.02 \). Bonferroni tests on each interval revealed that group LiCl (\( p \)'s < 0.05) displayed more passive drips on intervals 2, 4, and 6 than group saline or group cocaine ip. Only at interval 4 did cocaine sc (\( p \)'s < 0.05) show more passive drips than group saline or cocaine ip. For the CTA measure, the 4 x 4 ANOVA for each cumulative drinking measure revealed a significant main effect of group, \( F(3, 20) = 16.1, p < 0.001 \); Bonferroni tests overall revealed that group saline drank more saccharin solution overall than any other group (\( p \)'s < 0.025). As well, group LiCl had a greater overall CTA than group cocaine ip (\( p < 0.05 \)), but not cocaine sc. There was also a significant effect of time, \( F(3, 60) = 108.9, p < 0.001 \), and a group x time interaction, \( F(9, 60) = 6.3, p < 0.001 \); subsequent Bonferroni tests revealed that although the groups did not differ in the initial 30 min of intake, during interval 120
min all groups drank less saccharin than group saline ($p$’s < 0.001), but during intervals 240 and 360 min, only groups LiCl ($p$ < 0.001) and cocaine sc ($p$’s < 0.025) continued to drink significantly less than group saline.

**Motoric responses** The one-way ANOVA of the TR test motoric responses in experiment 2 revealed a significant group effect for the behaviors of active locomotion, $F(3, 20) = 3.8$, $p = 0.026$; face washing, $F(3, 20) = 7.6$, $p = 0.001$; head shakes, $F(3, 20) = 6.7$, $p = 0.003$; and forelimb flails, $F(3, 20) = 3.6$, $p = 0.03$. Subsequent Bonferroni comparison tests revealed that the group conditioned with cocaine ip were more active than the group conditioned with cocaine sc or LiCl ($p$’s < 0.05); no group differed from group saline in active locomotion. Groups LiCl ($p$ < 0.01) and cocaine sc ($p$ < 0.05) displayed significantly less face washing than group saline or cocaine ip ($p$’s < 0.01). Group LiCl displayed significantly fewer head shakes than group cocaine sc or cocaine ip ($p$’s < 0.05), but not group saline. Finally, group cocaine ip displayed more forelimb flails than group saline ($p$’s < 0.05), but no other groups differed (data not depicted).

**Experiment 3: delayed (30 min) context pairing with cocaine**

None of the aversive primary TR behaviors (gapes, chin rubs, and paw treads) nor activity measures revealed any differences between the cocaine and saline groups. The 2 x 6 mixed-factors ANOVA for gaping, chin rubbing, paw treading, and yawning were not significant. Therefore, the conditioned aversive effects evident in experiment 2 were the result of conditioning to the flavor, not the context. The mean distance traveled during each 5-min interval of the final test trial were entered into a 2 x 6 mixed-factors ANOVA which revealed only a
significant main effect of time, \( F(5, 70) = 4.6, p < 0.001 \), with all rats more active during the first 5-min interval (data not depicted).

**Experiment 4: delayed (10 min) saccharin pairing with cocaine or morphine**

Figure 5 presents the mean frequency or duration of the primary TR measures during each conditioning trial in experiment 4. Delayed access to cocaine and morphine (but to a lesser extent) produced conditioned gaping in rats even when the delay was only 10 min, and this effect (with cocaine) began after a single conditioning trial. However, the aversive behaviors of chin rubbing and paw treading were not produced. Both cocaine and morphine produced suppressed consumption in the CTA test and cocaine, but not morphine, enhanced passive dripping across trials.

For the behavior of gaping, the ANOVA across the five trials revealed a significant effect of group, \( F(2, 20) = 7.9, p = 0.003 \). Because we predicted that delayed access to a rewarding drug would produce aversive gaping, LSD comparison tests were used to reveal that both cocaine (\( p < 0.001 \)) and morphine (\( p = 0.028 \)) produced more gaping than saline conditioned groups. The trial effect, \( F(4, 80) = 12.4, p < 0.001 \), and the group x trial interaction, \( F(8, 80) = 5.3, p < 0.001 \), were also significant. Subsequent Bonferroni tests on each trial revealed that the cocaine conditioned group displayed more gaping than group saline on trials 2 (\( p < 0.05 \)), 4, and 5 (\( p’s < 0.001 \)), but group morphine did not differ from group saline on any trial. There were no significant group differences in chin rubbing or paw treading.

For the response of yawning, although all of the rats that yawned were in the cocaine group, the ANOVA revealed no significant effects across the trials. Since yawning was seen in
the cocaine conditioned group in experiment 2 following a 30-min taste exposure predominately during intervals 3–4 (minutes 10–20), it is likely that the lack of significant yawning in this group here is a function of the shorter duration (10 min) of exposure to the taste.

For tongue protrusions, the ANOVA revealed only a significant effect of trial, $F(4, 80) = 10.5, p < 0.001$. For passive dripping, the ANOVA revealed only a significant main effect of group, $F(2, 20) = 6.7, p = 0.006$; Bonferroni tests revealed that group cocaine displayed more passive dripping across trials than did group saline or group morphine ($p$’s < 0.025). Finally, the mean cumulative amount of saccharin consumed in the CTA test at 120, 240, and 360 min was entered into a 3 x 3 mixed-factors ANOVA, which revealed a significant effect of group, $F(2, 20) = 6.4, p = 0.007$; Bonferroni tests indicated that both group cocaine and morphine drank less saccharin overall than group saline. Groups cocaine and morphine did not significantly differ in their intake of saccharin solution. There was also a significant effect of interval of drinking, $F(2, 40) = 116.6, p < 0.001$, but no group by interval interaction.

The one-way ANOVA for each motoric behavior on the final TR test trial in experiment 4 revealed a significant group effect only for the behavior of forelimb flails, $F(2, 20) = 4.3, p = 0.028$, but subsequent Bonferroni tests revealed no significant difference among the groups.

Experiment 5: assessment of anxiogenic-like responding following exposure to saccharin previously paired with delayed access to cocaine

Rats given 10-s infusions of saccharin every min for 30 min prior to an injection of cocaine displayed anxiogenic-like responding when compared to the saline conditioned rats. This was not simply due to experience with cocaine, as the home cage rats injected with cocaine or
saline did not differ in the light–dark emergence test. Table 2 presents the mean (± SEM) number of seconds spent in the lit box. The rats in group Sac → Delayed Cocaine spent significantly less time in the lit box than the rats in group Sac → Delayed Saline, $t(12) = 2.35, p = 0.037$; however, group Home Cage Cocaine and Home Cage Saline did not differ from one another, $t(14) = 0.86$.

Table 3 presents the mean (± SEM) number of the TR behaviors scored during the 30-min session of saccharin exposure prior to the LD test. Group Delayed Cocaine displayed more gapes, $t(12) = 2.3, p < 0.5$; more yawns, $t(12) = 2.2, p < 0.05$; and fewer 2-s bouts of tongue protrusions, $t(12) = 2.6, p < 0.05$, than Group Delayed Saline. None of the other behaviors (or any motoric behaviors) differed among the groups.

**Discussion**

It has long been understood that rats will avoid a taste paired with rewarding drugs including cocaine (Booth et al. 1977; Goudie et al. 1977; Hunt and Amit 1987; Ferrari et al. 1991; Mayer and Parker 1993; Grigson 1997). Indeed, cocaine also produces the avoidance-like behaviors of suppressed tongue protrusions and enhanced passive dripping in the taste reactivity test (e.g. Parker 1993, 1995). However, when cocaine is immediately paired with a taste it does not produce a conditioned “aversion” characterized by the aversive taste reactivity measures of gaping, chin rubbing and paw treading, that are produced by emetic drugs, such as LiCl (Parker 1993, 1995). Here we replicate and extend this finding in rats that received daily conditioning trials with both ip and sc cocaine. During brief taste reactivity conditioning/testing trials with saccharin followed immediately by cocaine (either sc or ip), rats passively drip the taste from
their mouths and suppress ingestive tongue protrusions during intraoral delivery (replicating 
Parker 1993, 1995), as they show with a LiCl-paired flavor. Yet, unlike the LiCl-paired flavor,
rats do not display the aversive responses of gaping, chin rubbing or paw treading during an 
intraoral exposure to this cocaine- paired flavor. This was not likely due to the LiCl simply being 
more potent because while LiCl appeared to support a stronger CTA than cocaine in experiment 
1, it did not do so in experiment 2. Cocaine sc (20 mg/kg), but not LiCl, did enhance head shakes 
(often considered to be an aversive reaction) on the final test trial. On the contrary, like what was 
reported by Wheeler et al. (2008, 2011), rats given several saccharin exposures during each of 
four 30-min sessions prior to exposure to cocaine developed the aversive behaviors of gaping 
and chin rubbing; however, the remaining aversive behavior of paw treading was not apparent. 
Both LiCl and cocaine sc actually suppressed face washing (initially identified by Grill & 
Norgren, 1978a, and often included by others, as an aversive response). When the delay was 
decreased to 10 min in experiment 4, both sc cocaine and morphine produced gaping reactions, 
albeit the effect of cocaine was more robust than morphine, but neither produced the aversive 
reactions of chin rubbing or paw treading.

Another behavior that is rarely reported in a taste reactivity context is that of yawning, 
which has been reported as a withdrawal response to opiates and other drugs in animals 
(Nakamura-Palacios et al. 2002; Jaw et al. 1993; Schnur et al. 1992) and humans (Bickel et al. 
1988). Here we report that rats respond to delayed access to cocaine, but not immediate access to 
cocaine or immediate or delayed access to LiCl, with an orofacial yawning reaction (prolonged 
elongated vertical opening of the mouth followed by expiration of air) which is characteristically 
very different than gaping (wide opening triangular shape open mouth exposing bottom incisors).
This yawning response occurred between min 10 and 20 of the TR test during the 30-min delayed access to cocaine in experiment 2, but was not significant in experiment 4 with only a 10-min delay. Interestingly, yawning is a response that rats display to dopamine (D2, D3) agonists, such as quinpirole (e.g. Collins et al. 2005) or to serotonin 2c (5-HT2c) agonists, such as Loracserin (e.g. Serafine et al. 2015). Here this response emerged only in rats conditioned with long delayed (but not short delayed-10 min) access to cocaine (sc) and may represent a withdrawal response to cocaine as an indirect measure of a dysregulated dopamine and/or serotonin system.

The results of Experiment 5 provide an independent assessment of the conditioned aversive state produced by exposure to saccharin previously paired with delayed access to cocaine. Rats experiencing cocaine withdrawal display anxiogenic-like responding in preclinical models (Hu et al. 2016; de Oliveira Citó Mdo et al. 2012; Kupferschmidt et al. 2012), including the LD emersion test (Costell et al, 1990). Indeed, rats exposed to saccharin previously paired with delayed access to cocaine spent less time in the open lit box than rats exposed to saccharin previously paired with delayed access to saline, a pattern of anxiogenic-like responding. This difference was not simply the result of 24 h withdrawal from three daily exposures to cocaine, because home cage cocaine exposed rats did not differ from home cage saline exposed rats in time spent in the lit open box.

Our findings are therefore consistent with those of Wheeler et al. (2008, 2011) who suggested that such delayed exposure to cocaine produces an aversive state of withdrawal that becomes associated with the taste of saccharin and thus producing gaping reactions, as rats also show to a flavor paired with opiate withdrawal (McDonald et al. 1997). Several lines of evidence
support such a contention. Using fast-scan voltammetry, Wheeler et al. (2011) observed decreased mesolimbic dopamine concentrations to a taste cue that signaled delayed cocaine availability, but this switched to elevated mesolimbic dopamine concentrations to taste cues signaling imminent cocaine delivery in a self-administration session. As well, under conditions of delayed access to cocaine, NAc neurons display a quinine-like (opposite of a sucrose-like) excitability pattern (Roitman et al. 2005; Wheeler and Carelli 2009) during saccharin exposure. Interestingly, the excitatory response profile and the aversive taste reactivity are inversely correlated with the latency to make the first press for cocaine. These findings suggest that the switch in NAc activity from inhibitory to excitatory during infusions of the cocaine-paired taste reflects the learned association between the taste and the negative affective state of withdrawal that drives the increased motivation to consume cocaine when available. Furthermore, rats given 20-min access to saccharin which had been paired with morphine displayed suppressed DA signaling compared with rats conditioned with saline (Grigson and Hajnal 2007). Finally, presentation of a taste cue predictive of delayed access to cocaine produced an elevated intracranial self-stimulation threshold (Wheeler et al. 2011). These findings collectively indicate a pronounced dampening of the DA system during exposure to a taste that signaled delayed cocaine or morphine availability.

Carelli and West (2014) hypothesize that the increased motivated behavior for cocaine following this learned association may be a consequence of the development of a negative affective state that is a consequence of delayed drug availability. Koob and colleagues (Ahmed and Koob 1998) argue that chronic cocaine self-administration alters the rat’s hedonic set point, reducing responsiveness to rewarding stimuli, as a modified version of the opponent process.
theory (Solomon and Corbit 1974). This allosteric regulation reduces the hedonic set point by increasing the function of the brain “anti-reward” system, thereby increasing tolerance to the hedonic effects of cocaine (see also Siegel and Ramos 2002). Wheeler et al. (2008) found that this negative state may be alleviated by drug loading; that is, rats that show the strongest aversions to delayed cocaine-paired taste also showed the greatest self-administration of cocaine. Thus, during this waiting period, the rat experiences an aversive state that may include the onset of conditioned anxiety, craving and/or withdrawal (a cocaine-need state). Nyland and Grigson (2013) provided some direct evidence for a withdrawal interpretation. Following several days of pairing taste exposure with the delayed opportunity to self-administer cocaine, rats were exposed to the cocaine associated taste followed by an injection of naloxone which can precipitate withdrawal, not only from morphine, but also from cocaine, as measured by body weight loss. The cocaine group had a significant loss in body weight 2 h after naloxone administration and the greater the weight loss, the greater the subsequent cocaine self-administration. These findings suggest that avoidance of the taste may result from the development of an aversive conditioned withdrawal state that develops when the taste cue comes to predict the delayed availability of the drug.

The failure to see conditioned aversive responses to saccharin when paired with immediate access to cocaine cannot simply be attributed to less exposure to the saccharin, because in experiment 3, the rats received the same amount of saccharin exposure as in experiment 1 (2 ml), but the saccharin signaled a waiting period of 10 min. Thus, the saccharin became a cue for the delayed availability of cocaine and morphine in experiment 3 producing gaping reactions. This finding extends the effect to another rewarding drug and supports
Grigson’s (1997) model of reward comparison, as well. Indeed, as has also been shown by Colechio et al (2014) the development of a negative affective state produced by delayed availability of cocaine appears to develop even after only a single conditioning trial.

There is an issue, however, that remain somewhat unclear. If saccharin acquires conditioned aversive effects because it comes to predict the delayed delivery of a highly-desired drug, then it is not clear why sc cocaine, which was ineffective in producing a conditioned place preference (Mayer and Parker 1993, see also Tzschentke 1998), is more effective in producing a negative conditioned affective state than is ip cocaine which is more effective in producing a CPP. As well, sc cocaine was also more effective than 10 mg/kg sc morphine in producing conditioned aversion with a 10-min delay, yet morphine consistently produces a CPP at this dose at the sc route of administration (e.g., Mueller et al. 2002; Tzschentke 1998).

Here we have presented each of the behaviors separately that were initially identified in the TR test by Grill and Norgren (1978a). Many researchers in the field have taken shortcuts of only measuring certain of these behaviors, such as gaping, or combining across selective behaviors providing total aversive score. For instance, the behavior of face washing has been combined with gaping as an aversive measure; however, the results of experiments 1c and 2 clearly show that face washing is decreased (not increased) by pairings of saccharin with LiCl or delayed access to sc cocaine. Clearly the responses of face washing, head shaking, forelimb flailing, and general activity are not consistently aversive responses, as seen here and discussed by Parker (1995). It will be important for future researchers to avoid composite scores that may not reflect a common process.
Table 1. Definition of Taste Reactivity Test Behaviors Scored

<table>
<thead>
<tr>
<th>Type of behavior</th>
<th>Behavior</th>
<th>Characteristics</th>
<th>Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aversive</td>
<td>Gape</td>
<td>Wide, triangular opening of the mouth, retraction of the corners of the mouth, exposing incisors</td>
<td>Frequency</td>
</tr>
<tr>
<td>Aversive</td>
<td>Chin rub</td>
<td>Sustained contact of chin with floor or walls of the chamber</td>
<td>Frequency</td>
</tr>
<tr>
<td>Aversive</td>
<td>Paw tread</td>
<td>Quick movement of forepaws on the floor of the cage, alternating paws-while not moving forward</td>
<td>Frequency</td>
</tr>
<tr>
<td>Withdrawal-like response</td>
<td>Yawn</td>
<td>Elongated vertical opening of the mouth without retraction of the corners of the mouth followed by expiration of air</td>
<td>Frequency</td>
</tr>
<tr>
<td>Motor</td>
<td>Active locomotion</td>
<td>Forward movement -one forepaw in front of other</td>
<td>Duration (s)</td>
</tr>
<tr>
<td>Motor</td>
<td>Rear</td>
<td>Lifting of forepaws from the chamber floor, standing on hindlimbs</td>
<td>Duration (s)</td>
</tr>
<tr>
<td>Motor</td>
<td>Face wash</td>
<td>Grooming of the face with forelimbs</td>
<td>Duration (s)</td>
</tr>
<tr>
<td>Motor</td>
<td>Head shake</td>
<td>Rapid side to side movement (shaking) of the head</td>
<td>Frequency</td>
</tr>
<tr>
<td>Motor</td>
<td>Forelimb flail</td>
<td>Swinging of the forelimbs</td>
<td>Frequency</td>
</tr>
<tr>
<td>Positive hedonic</td>
<td>Tongue protrusion</td>
<td>2-s bouts of rhythmic protrusions of the tongue</td>
<td>Frequency</td>
</tr>
<tr>
<td>Avoidance-like</td>
<td>Passive drips</td>
<td>Dripping of solution from the mouth</td>
<td>Frequency</td>
</tr>
</tbody>
</table>
### Table 2. Mean (+SEM) seconds spent in open lit box in experiment 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (±SEM) seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group Sac → Delayed Saline (n = 7)</td>
<td>183.6 (±5.2) s</td>
</tr>
<tr>
<td>Group Sac → Delayed Cocaine (n = 7)</td>
<td>165.3 (±5.8) sec</td>
</tr>
<tr>
<td></td>
<td>*p &lt; 0.05</td>
</tr>
<tr>
<td>Group Home Cage Saline (n = 8)</td>
<td>107.1 (±8.7) s</td>
</tr>
<tr>
<td>Group Home Cage Cocaine (n = 8)</td>
<td>110.7 (±18.4) s</td>
</tr>
</tbody>
</table>

### Table 3. Mean (+SEM) number of TR behaviors during 30-min test infusion in experiment 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group Sac → Delayed Saline</td>
<td></td>
</tr>
<tr>
<td>Gape</td>
<td>0.0</td>
</tr>
<tr>
<td>Chin Rub</td>
<td>0.0</td>
</tr>
<tr>
<td>Yawn</td>
<td>0.6 (±0.1)</td>
</tr>
<tr>
<td>Paw Tread</td>
<td>0.0</td>
</tr>
<tr>
<td>Passive Drip</td>
<td>0.0</td>
</tr>
<tr>
<td>Tongue Protrusions</td>
<td>180.6 (±27.1)</td>
</tr>
<tr>
<td>Group Sac → Delayed Cocaine</td>
<td></td>
</tr>
<tr>
<td>Gape</td>
<td>29.4 (±12.8) *</td>
</tr>
<tr>
<td>Chin Rub</td>
<td>8.6 (±5.4)</td>
</tr>
<tr>
<td>Yawn</td>
<td>5.4 (±2.2) *</td>
</tr>
<tr>
<td>Paw Tread</td>
<td>0.6 (±0.4)</td>
</tr>
<tr>
<td>Passive Drip</td>
<td>3.6 (±2.6)</td>
</tr>
<tr>
<td>Tongue Protrusions</td>
<td>99.6 (±16.5) *</td>
</tr>
</tbody>
</table>

* p < 0.05
Mean (±SEM) frequency or duration (s) of primary TR measures and CTA in experiment 1a with immediate sc cocaine. Asterisks indicate a group difference from saline; *p < 0.05, **p < 0.01, ***p < 0.001.
Mean (±SEM) frequency or duration (s) of each of primary TR measures and CTA in experiment 1b with immediate ip cocaine. Asterisks indicate a group difference from saline; *p < 0.05, **p < 0.01, ***p < 0.001.
Mean (±SEM) frequency or duration of primary TR measures and CTA in experiment 1c with immediate LiCl (50 mg/kg, ip). Asterisks indicate a group difference from saline; *p < 0.05, **p < 0.01, ***p < 0.001.
Mean (±SEM) frequency or duration (s) of primary TR measures and CTA in experiment 2 with 30 min delayed cocaine (20 mg/kg, either sc or ip) and LiCl (50 mg/kg, ip). Asterisks indicate a group difference from saline; *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 5

Mean (±SEM) frequency or duration primary TR measures and CTA in experiment 4 with 10 min delayed cocaine (20 mg/kg, sc) or morphine (10 mg/kg, sc). Asterisks indicate a group difference from saline; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 
CHAPTER 3

Additional experiments:

Can a brief (2-min) saccharin infusion preceding a delay (30-min) to cocaine or lithium chloride administration result in conditioned gaping in male Sprague-Dawley rats?

AND

Potential of Ondansetron, SCH 23390 or Oleoyl Glycine pre-treatments to interfere with the establishment of conditioned gaping produce by delayed cocaine in male Sprague-Dawley rats
Introduction

Considerable evidence indicates that cocaine abuse produces negative affect that can precipitate craving and relapse in humans and animal models (e.g. Koob and LeMoal 1997; Solomon and Corbit 1974; Wheeler et al. 2008). This negative affective state has been demonstrated with the establishment of the conditioned gaping reaction (Grill and Norgren 1978a) in the taste reactivity (TR) test (Wheeler et al. 2008; Wheeler et al. 2011). Specifically, rats who experience brief intraoral infusions of saccharin solution throughout a waiting period for cocaine administration develop the behavioural reaction of gaping during the delay, indicating a conditioned aversion to the flavour (Wheeler et al. 2008). It is hypothesized that the development of conditioned aversion is due to an opponent process effect (Solomon and Corbit 1974), meaning a saccharin cue that signals delayed availability of cocaine can elicit aversive feelings of craving or withdrawal in anticipation of the imminent euphoric and reinforcing effects of cocaine.

Although immediate access to cocaine following a brief 2-min infusion of saccharin does not produce conditioned gaping reactions (Parker 1995; Guenther et al. 2018) and administration of multiple brief exposures of saccharin for 10-30 minutes paired with delayed cocaine does result in conditioned gaping upon re-exposure to the saccharin (eg. Wheeler et al. 2008; Wheeler et al. 2011), it is unclear whether saccharin needs to be administered intermittently over the entire delay or if the same conditioned gaping effect can be observed even if a brief saccharin administration precedes the delay. Specifically, it is not known whether saccharin can be conditioned to be aversive if it is administered for a 2-minute infusion period, as is done in immediate procedures, followed by a 30-minute delay to cocaine where no saccharin
administration is present. To answer this question, rats were infused with saccharin for a 2-min period in the TR chamber followed 30-min later by cocaine (20 mg/kg, sc). To determine the role of the context mediating the development of conditioned gaping during the waiting period, half of the rats remained in the TR context (context group) during the delay and half of the rats were returned to their home cage (home cage group) during the delay. As a positive control group, another group of rats were administered a low dose of LiCl (50 mg/kg, ip) 30-min following a 2-min exposure to saccharin. Although it is known that rats will avoid consuming a flavor paired with LiCl over delays as long as 6-12 h (Garcia, Hankins and Rusiniak 1974), to the best of our knowledge it is not known if rats will avoid consuming a flavor paired with rewarding drugs over a delay. As well, it has not been determined if conditioned gaping produced by LiCl can be produced over a delay when LiCl is presented at a time greater than 15 minutes following a brief (3-min) infusion of saccharin (Sticht et al. 2015).

Next, additional experiments evaluated the potential of pre-treatments to interfere with the establishment of the conditioned aversive state produced by delayed (10 min) access to cocaine that is paired repeatedly with intermittent, brief (10 sec/min at the rate of 1.2 ml/min-giving a total of 2 ml saccharin total) access to saccharin (Guenther et al. 2018). As mentioned, it has been suggested that the aversive state produced by delayed cocaine is similar to cocaine withdrawal or craving (Carelli and West 2014). However, the conditioned gaping model is an established animal model of nausea, with much research demonstrating that gaping occurs when a flavour is paired with an illness inducing agent (Parker 2014). In traditional TR experiments where rats receive a 2-min infusion of a flavour followed immediately by administration of a drug, only emetic drugs or drugs that produce nausea in humans have been shown to elicit this
conditioned gaping response (see Parker 2014). Indeed, when cocaine is immediately preceded by a 2-min intraoral infusion of saccharin in a typical TR experiment, it fails to produce the conditioned gaping reaction over repeated trials (Parker 1993; Guenther et al. 2018). However, when the flavour exposure is conditioned under the schedule described by Wheeler et al. (2008) – specifically, multiple brief exposures (10 sec/every min) to saccharin for a delay (10-30 min) prior to delivery of cocaine or morphine – saccharin eventually elicits the aversive response of conditioned gaping (Guenther et al. 2018; Carelli and West 2014; Wheeler et al. 2008). With such a procedure saccharin acquires the capacity to signal a waiting period for delayed administration of cocaine, which possibly serves as an aversive state stimulus. Whether or not this aversive state includes the sensation of nausea is not known.

Limebeer and Parker (2000) demonstrated that ondansetron, a 5-HT₃ receptor antagonist that is commonly prescribed to patients for the management of nausea and vomiting, interferes with the establishment of LiCl-induced conditioned gaping in rats, without affecting conditioned taste avoidance. We first examined the potential of ondansetron to interfere with the establishment of conditioned gaping produced by delayed access to cocaine. If the conditioned gaping produced by delayed cocaine is due to nausea, ondansetron should prevent the establishment of gaping, but not interfere with taste avoidance.

Additionally, we examined the potential of the dopamine D1 receptor antagonist SCH 23390 to interfere with the establishment of delayed cocaine-induced conditioned gaping. SCH 23390 is highly selective and has a high binding affinity for the D1 receptor, making it a potent antagonist at this receptor (Bourne 2001). D1 receptors are implicated in reward signaling in the brain and are important in early drug use for the development of addiction (Self 2010). Previous
research has shown that knockout of D1 receptors interferes with the establishment of cocaine self-administration and that infusions of the D1 receptor antagonist SCH 23390 into the nucleus accumbens interferes with cocaine-induced conditioned place preference (Self 2010; Baker et al. 1998). Because D1 receptors have been shown to mediate establishment of cocaine self-administration, it is hypothesized that administration of SCH 23390 will also interfere with the establishment of conditioned gaping reactions to saccharin paired with delayed cocaine. This finding would indicate that the rewarding properties of cocaine are implicated in the establishment of conditioned gaping to saccharin in the delayed procedure.

Finally, we evaluated the potential of a newly discovered endogenous fatty acid amide, oleoyl glycine (OlGly), to interfere with the establishment of conditioned gaping produced by delayed access to cocaine. This compound has been shown to have anti-nausea and anti-emetic effects (Rock et al. in preparation), as well as the capacity to interfere with the development of nicotine-addiction and opiate withdrawal (Donvito et al. 2019; Petrie et al. 2019).

Methods

Subjects

The subjects were 76 naïve male Sprague-Dawley rats were obtained from Charles River Laboratories (St Constant, Quebec). The rats were between 250-300g in weight on the first conditioning trial. All rats were individually housed in home cages made of opaque white plastic (48 x 26 x 20 cm), containing bed-o-cob bedding from Harlan Laboratories, Inc. (Mississauga, Ontario), a brown paper towel, and Crink-l'Nest™ from The Andersons, Inc. (Maumee, Ohio). All rats were maintained in a colony room with an ambient temperature of 21°C and a 12/12 hour
light-dark schedule (lights off at 7 AM), and behavioural testing was conducted during the dark phase of the light cycle. Rats were maintained on food (Highland Rat Chow) and water ad-libitum.

**Surgery**

Rats were surgically implanted with an intraoral (IO) cannula 1 week prior to conditioning. Implantation of the cannula was done while rats were under isoflurane anesthesia according to the procedures previously described (Limebeer et al. 2010). Rats underwent 3 days of post-surgical monitoring beginning the day following surgery.

**Drugs**

Cocaine was mixed at a concentration of 1.5 mg/ml in solution with saline (SAL) for subcutaneous (sc) injection in order to prevent skin necrosis. As well, the injection site was changed on a daily basis to avoid skin necrosis. A dose of 20 mg/kg cocaine was used and was administered at 13.3 ml/kg. LiCl was prepared as a 0.15 M solution in sterile saline water and administered at a volume of 8 ml/kg ip (50 mg/kg). Ondansetron was mixed in SAL at a concentration of 0.5 mg/ml and administered sc at 1 ml/kg (0.5 mg/kg). SCH 23390 was mixed in SAL at a concentration of 0.05 mg/ml and administered sc at 1 ml/kg (0.05 mg/kg). OlGly was dissolved in a vehicle mixture of ethanol, Tween 80 and physiological saline in a 1:1:18 ratio; OlGly was first dissolved in ethanol, Tween 80 was added to the solution, then ethanol was evaporated off with a nitrogen stream. Finally, physiological saline was added to finish with OlGly at a concentration of 5 mg/ml.
Apparatus

For TR measures, the rats were placed in a clear Plexiglas box (22.5 x 26 x 20 cm) with an opaque lid, sitting on top of a clear glass-topped table. A mirror was located under the chamber at a 45° angle in order to allow viewing of the rat ventral surface. The chamber was located in a dark room next to a 25-W light source. There was a video camera (Sony DCR-HC48; Henry’s Cameras, Waterloo, ON, Canada) placed in front of the mirror to allow the trials to be recorded and scored at a later time. Trials were scored by an observer blind to the experimental groups using “The Observer” (Noldus Information Technology Inc., Leesburg, VA, USA).

Procedure

Experiment 1: Brief (2 min) saccharin exposure followed by delay (30 min) to cocaine or LiCl administration

All rats were adapted to the taste reactivity chamber three days prior to the first conditioning trial. During adaptation, the rat was placed in the reactivity chamber, their cannula attached to an infusion pump (Model KDS100, KD Scientific, Holliston, MA, USA) for fluid delivery. For adaptation, water was infused into the intraoral cannula at a rate of 1ml/min for a total of 2 min.

Three days following adaptation, the first conditioning trial began. On each of 5 conditioning trial/testing trials, the rat was placed in the taste reactivity chamber, its cannula was attached to the pump for the infusion of 0.1% saccharin at a rate of 1 ml/min for 2 min. The timer started when the rat made an orofacial response and stopped at 2 min. The trial was recorded for
scoring at a later time. The rats in the home cage delay conditioning groups were removed from the chamber and returned to their home cage; 30 min later they were given a sc injection of saline \((n = 8)\), 20 mg/kg cocaine \((n = 8)\) or 50 mg/kg LiCl \((n = 8)\) on trials 1-4. The rats in the context delay conditioning groups remained in the TR chamber for 30 min prior to being given a sc injection of saline \((n = 7)\), 20 mg/kg cocaine \((n = 8)\) or 50 mg/kg of LiCl \((n = 6)\) on trials 1-4.

Among the home cage conditioned rats only, at 15:00 on the day after the testing, the water bottles were removed from each rats cage. At 8:00 on the following day, rats were given 0.1% saccharin solution in graduated tubes and the amounts consumed at 120 min was measured as a measure of conditioned taste avoidance (CTA).

**Experiment 2: Effect of ondansetron, SCH 23390 and oleoyl glycine on conditioned gaping and CTA produced by delayed cocaine**

All rats were adapted to the taste reactivity chamber three days prior to the first conditioning trial. During adaptation, the rat was placed in the taste reactivity chamber and its cannula was attached to an infusion pump (Model KDS100, KD Scientific, Holliston, MA, USA) for fluid delivery. For adaptation, water was infused into the intraoral cannula at a rate of 1.2 ml/min for 10 seconds every minute for 10 minutes for a total delivery of 2 ml.

Three days following adaptation, the first conditioning trial began. On each of 4 conditioning trials, the rat was given a pre-treatment injection of saline \((n = 9; \text{sc})\), ondansetron \((n = 8; 0.5 \text{ mg/kg, sc})\), or SCH 23390 \((n = 7; 0.05 \text{ mg/kg, sc})\) 20 min prior to being placed in the TR chamber or OlGly \((n = 7; 5 \text{ mg/kg, ip})\) 10 min prior to being placed in the TR chamber. The rat’s cannula was then attached to the pump and 0.1% saccharin was infused according to the
same schedule as water during adaptation (1.2 ml/min for 10 sec every min for 10 min). The timer started when the rat made an orofacial response and stopped at 10 min. The trial was recorded for scoring at a later time. Immediately following the 10-minute TR trial, rats were injected with 20 mg/kg sc cocaine and returned to their home cage. The final test trial (trial 5) was carried out as in conditioning, however rats did not receive a pre-treatment or cocaine injection.

At 15:00 on the day after the testing, the water bottles were removed from each rats cage. At 8:00 on the following day, rats were given 0.1% saccharin solution in graduated tubes and the amounts consumed at 120 min was measured as a measure of conditioned taste avoidance (CTA).

**Results**

*Experiment 1: Brief (2 min) saccharin exposure followed by delay (30 min) to cocaine or LiCl administration*

Regardless of the contextual conditions during the delay, a 2-min infusion of LiCl-paired saccharin produced conditioned gaping, but not cocaine-paired saccharin, despite equal avoidance of LiCl and cocaine paired saccharin in the CTA test of the home cage conditioned rats. Figure 1 presents the mean number of gapes displayed by the rats that spent the 30-min delay in the home cage (upper section) and in the TR chamber during the delay (lower section). The insert in the upper section of Figure 1 presents the result of the 2 h CTA test. The 3 x 5 mixed factors ANOVA of the gaping data group remaining in the home cage during the delay revealed a significant effect of group, $F(2, 21) = 10.4, p < 0.001$; trial $F(4, 84) = 12.2; p < 0.001$;
and a group x trial interaction, $F(8, 84) = 4.7; p < 0.001$. For the main effect of group, Bonferroni tests revealed that Group LiCl displayed more gaps overall than Group cocaine ($p < 0.05$) or saline ($p < 0.001$). Bonferroni tests for each trial revealed that Group LiCl showed more gaping than Group saline ($p's < 0.001$) and Group cocaine ($p < 0.05$) on trials 2, 4 and 5. Group cocaine and saline did not differ on any trial. The $3 \times 5$ mixed factors ANOVA of the gaping data for the group that remained in the conditioning context during delay also revealed significant effects of group, $F(2, 18) = 13.5; p < 0.001$; trial, $F(4, 72) = 13.3; p < 0.001$; and a group x trial interaction, $F(8, 72) = 5.9, p < 0.001$. For the main effect of group, Bonferroni tests revealed that overall Group LiCl showed more gaping ($p's < 0.01$) than cocaine or saline which did not differ. As well, Bonferroni tests for each trial revealed that Group LiCl showed more gaping than Group saline ($p's < 0.01$) or cocaine ($p's < 0.06$) on trials 3-5; Groups cocaine and saline did not differ on any trial. For the 2 h CTA test, a single factor ANOVA revealed that Group Saline drank significantly ($p < 0.001$) more saccharin than Group cocaine or LiCl, $F(2,23) = 97.5; p < 0.001$, which did not differ from one another.

**Experiment 2. Effect of ondansetron, SCH 23390 and oleoyl glycine on conditioned gaping and CTA produced by delayed cocaine administration.**

On trials 2 and 3, ondansetron, SCH 23390 and OlGly all interfered with conditioned gaping produced by delayed cocaine; however, this initial interference was overcome with additional conditioning trials. The D1 antagonist SCH 23390 also dramatically suppressed locomotor activity. The pre-treatments did not modify the CTA suggesting that they did not modify learning the association between saccharin and delayed cocaine. The number of gapes
(upper section of Figure 2) and seconds of active locomotion (bottom section of Figure 2) were entered into 3 x 5 mixed factors ANOVA. The gaping data revealed a significant effect of trial, $F(4, 108) = 20.8; p < 0.001$, and only marginal effects of group x trial, $F(12, 108) = 1.6; p < 0.10$, and group, $F(3, 270) = 2.4; p < 0.10$. Subsequent single factor ANOVAs for each trial revealed a significant group effect only for trial 2, $F(3, 27) = 4.3; p < 0.025$, and trial 3, $F(3, 27) = 4.3; p < 0.025$. Subsequent Least Significant Difference (LSD) tests revealed that on both trials 2 and 3, Group VEH displayed significantly more gaping than any other group, which did not differ from one another. For active locomotion, the ANOVA revealed significant effects of trial, $F(4, 108) = 9.2; p < 0.001$; group x trial, $F(12, 108) = 6.4; p < 0.001$ and a group effect, $F(3, 27) = 10.3; p < 0.001$. Subsequent single factor ANOVAs revealed significant effects of group on each trial except the test trial. LSD posthoc comparison tests revealed that on Trial 1, Group SCH 23390 was less active ($p < 0.001$) and OlGly was more active ($p < 0.001$) than VEH or ondansetron. On trials 2-4, Group SCH 23390 was less active than all other groups ($p < 0.001$). The 2 h CTA test, depicted in the upper insert of Figure 2, revealed no significant differences among the groups.

Discussion

The first experiment demonstrated that a 30-min delay following a brief exposure to saccharin paired with LiCl, but not cocaine produced conditioned gaping reactions. This difference was apparent even though both delayed cocaine and delayed LiCl produced an equivalent CTA. This finding suggests that for delayed access to cocaine to produce conditioned gaping reactions, temporal proximity is needed between saccharin and cocaine administration.
Similar results were seen when the delay occurred either in the TR context or in the home cage. It is likely that delayed LiCl and cocaine are causing different physiological states which may influence their association with saccharin. It is established that LiCl produces nausea and vomiting when administered (Parker 2014), therefore conditioned gaping to saccharin paired with delayed LiCl in this paradigm is likely due to the aversive state of nausea. Contrarily, as suggested by Wheeler et al. (2008) the association between intermittently presented saccharin over a delay and cocaine may result in saccharin signaling an aversive negative state over the period during which the rats must wait for administration of cocaine, a rewarding drug. We have shown that LiCl and cocaine are equivalent in producing conditioned gaping when saccharin is administered intermittently over the 30-min delay (Guenther et al. 2018).

The second experiment demonstrated that each of the pre-treatments attenuated the establishment of a conditioned aversion to delayed access to cocaine during the early conditioning trials, but the suppressive effect was overcome with additional conditioning trials. However, the effects of SCH 23390 may have been the result of a dramatic general suppression of locomotion. Ondansetron is a classical anti-emetic drug that has been shown to interfere with LiCl-induced conditioned gaping in rats (Limebeer and Parker 2000). It has also been suggested to reduce anxiety in animals experiencing cocaine withdrawal (Costall et al. 1989). It is unclear whether the ability of ondansetron to interfere with the establishment of gaping in early conditioning trials is due to its anti-nausea or anti-anxiety effects, therefore future studies should examine this further. SCH 23390 is highly selective and has a high binding affinity for the D1 receptor, making it a potent antagonist at this receptor (Bourne 2001). Since D1 receptors are implicated in early drug use for the development of addiction (Self 2010), our results suggest that
they may be important for producing at least the early stages of the aversive effects of delayed access to cocaine. OlGly is a newly discovered anandamide-like compound that acts at cannabinoid 1 (CB1) receptors to reduce morphine withdrawal reactions (Petrie et al. 2018). Our results suggest that it may reduce the early stages of the establishment of cocaine withdrawal as well. However, the suppressive effect of each of these pre-treatment drugs was overcome with additional conditioning exposure. Indeed, by the test trial (trial 5) with no pre-treatment drug “on board”, none of the groups differed in the strength of gaping reactions. These results must be interpreted with caution and only serve as a preliminary assessment of the mechanism underlying the establishment of conditioned gaping produced by delayed access to cocaine. Future research evaluating the effectiveness of several doses of each of the pre-treatment drugs is necessary.

Limitations

We hypothesized that ip cocaine administration would result in more conditioned gaping when compared to sc administration because of the rewarding properties of ip cocaine (Mayer and Parker 1993), however we found the opposite result in our 30-min delayed paradigm. Similarly, we found that a highly rewarding dose of morphine (10 mg/kg, sc) produced less conditioned gaping in the 10-min delayed paradigm compared to a non-rewarding dose of cocaine (20 mg/kg, sc). If saccharin is conditioned to be aversive because it is a cue that predicts delayed reward delivery (Wheeler et al. 2008), then it is unclear why sc cocaine is more effective at producing conditioned gaping than ip cocaine or morphine. These results provide a limitation to the original interpretation that conditioned gaping to saccharin paired with delayed cocaine is due to delayed reward, as suggested by Wheeler et al. (2008).
Next, interpretation of the result that pre-treatment injections of the D1 receptor antagonist SCH 23390 interfered with the establishment of conditioned gaping to saccharin paired with delayed cocaine should be interpreted with caution. Specifically, active locomotion – a measure of activity in the TR paradigm – was drastically reduced in this group. It is possible that suppression of conditioned gaping is caused by reduced activity following administration of SCH 23390, as opposed to interference with the development of an association between saccharin and the rewarding effects of cocaine.

Finally, only one dose of each pre-treatment drug (ondansetron, SCH 23390 and OlGly) was tested in our delayed cocaine paradigm. Therefore, results from this experiment should only be considered as a preliminary assessment of the mechanism underlying the establishment of conditioned gaping to saccharin paired with delayed cocaine. Future research should evaluate the effectiveness of several doses of each pre-treatment drugs. Additionally, future testing with DA receptor antagonists should consider doses that will not interfere with activity measures.
Mean (±SEM) frequency of gaping in the TR test and CTA in Experiment 1 with 2-min saccharin infusion followed by a 30-min delay to cocaine (20 mg/kg sc) or LiCl (50 mg/kg ip). Asterisks indicate a group difference from saline; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 
Figure 2

Mean (±SEM) number of gapes and duration of active locomotion (s) displayed by the various pre-treatment groups across conditioning and testing trials. The insert represents the mean ml of saccharin consumed during the subsequent 2 h CTA. Asterisks indicate a group difference from saline; *p < 0.05, **p < 0.01, ***p < 0.001.
CHAPTER 4

General Conclusion

The experiments in Chapter 2 show that when cocaine is administered immediately following a 2-min infusion of saccharin, rats do not display conditioned gaping upon subsequent exposures to saccharin. Contrarily, when cocaine is administered following a delay (10 or 30 min) during which rats are being intraorally infused with saccharin every minute for 10 seconds, both sc and ip cocaine are able to produce the aversive gaping reaction after repeated conditioning trials.

The results from the immediate procedure replicate the findings of Parker (1993), that when cocaine is administered immediately following a 2-min infusion of sucrose over repeated conditioning trials, there is no development of the aversive behaviour of gaping. Similarly, in this initial study Parker (1993) reports a significant suppression of ingestive behaviours, and an increase in passive drips during the taste reactivity (TR) test. A suppression of tongue protrusions and the occurrence of passive drips was also observed in our study, indicating that while saccharin paired with immediate cocaine does not result in the development of taste aversion, it does result in taste avoidance, as seen by the avoidance-like behaviours in the TR test. Additional support for this is seen in the conditioned taste avoidance (CTA) test, where rats avoided saccharin that had been paired with immediate cocaine (20 mg/kg ip). Results were compared to administration of a moderate dose of lithium chloride (LiCl; 50 mg/kg) following a 2-min infusion of saccharin, a drug known to produce conditioned gaping in the TR paradigm. As expected, conditioned gaping was elicited by saccharin paired with immediate LiCl, unlike the sc or ip cocaine. However, similar to cocaine, LiCl paired saccharin causes a reduction in the
ingestive behaviour of tongue protrusions and elicited passive drips. This finding has been well documented in the literature. Although rewarding drugs fail to produce aversive behaviours in the TR paradigm, they do result in taste avoidance in a bottle-test to a flavour with which they have been paired, to the same degree as drugs that produce aversive behaviours in the TR paradigm (Parker and Mayer, 1993).

In contrast, when cocaine is administered following a 30-min delay during which rats are being intermittently intraorally infused saccharin every minute for 10 seconds, both sc and ip cocaine (20 mg/kg) are able to elicit the aversive gaping reaction after repeated conditioning trials. When comparing routes of administration, sc cocaine was able to elicit more conditioned gaping than ip cocaine, however neither route produced other aversive behaviours in the TR test (specifically chin rubbing and paw treading). Similar to the immediate cocaine pairing results, saccharin paired with delayed sc or ip cocaine produced the avoidance-like behaviour of suppressed tongue protrusions, however only sc resulted in passive dripping. Despite this, route of administration did not affect CTA, as both groups avoided saccharin consumption equally. When comparing the delayed cocaine TR profile to that of the aversive drug LiCl, we see that in addition to producing conditioned gaping, LiCl paired saccharin was also able to produce the aversive behaviours of chin rubbing and paw treading. The absence of chin rubbing and paw treading in the cocaine groups may indicate differing subjective states. LiCl is a well-known emetic agent that produces nausea, which is commonly measured with the aversive TR behaviours of gaping, paw treading and chin rubbing. The observation that cocaine is able to produce gaping, but not the other two aversive behaviours may indicate an internal state that differs from nausea.
An experiment presenting a shorter (10-min) delay to cocaine administration was conducted in order to provide a control for the amount of saccharin exposure on development of conditioned aversive responses. Specifically, the total amount of saccharin infused in the immediate (2-min) procedure was 2 ml, whereas the amount of saccharin delivered intermittently for 10 sec every minute over 30 min was 6 ml. A 10-min delay allowed for the delivery of a total of 2 ml (when administered for 10 sec every minute over 10 min), as in the immediate procedure, while still establishing a delay to cocaine administration. When the delay between saccharin administration and cocaine (20 mg/kg sc) was decreased to 10-min, saccharin still elicited the aversive behaviour of gaping after repeated conditioning trials. This finding highlights the importance of a delay with intermittent saccharin exposure between initial saccharin exposure and cocaine administration, as opposed to the volume of saccharin administered. In this 10-min delayed procedure, morphine administration was also able to produce conditioned gaping after repeated conditioning trials. Although conditioned gaping was more robust when saccharin was paired with delayed cocaine in this procedure, it still indicates that this conditioned aversive effect is generalizable to other rewarding drugs, specifically morphine.

To better understand the aversive state that develops to saccharin paired with delayed cocaine, we subjected rats to the Light-Dark (LD) Emergence test – a measure of anxiety – immediately following presentation of delayed-cocaine paired-saccharin. These rats displayed less time in the open lit box than rats exposed to saccharin previously paired with delayed saline, indicating anxiogenic-like responding. The increased anxiety-like behaviour in cocaine rats was not simply the result of 24 h withdrawal from four daily exposures to cocaine, because home
cage cocaine exposed rats who did not experience saccharin-delayed cocaine pairings did not differ from home cage saline exposed rats in time spent in the lit open box.

Wheeler et al. (2008) proposes that the conditioned gaping reaction that emerges from repeated pairing of saccharin with delayed cocaine is an indication of a negative affective state. It is well known that cocaine use is accompanied by negative affective feelings such as dysphoria, irritability and anhedonia, and that these can emerge follow exposure to a cocaine related cue (Koob and LeMoal 1997). They suggested that the saccharin serves as a cocaine related cue that signals a waiting period for coming cocaine, and that this cue is capable of producing a negative affective state in anticipation of coming cocaine, measured with conditioned gaping (Wheeler et al, 2008). Furthermore, they believe this negative affective state represents a state of cocaine withdrawal that subsequently induces craving. Rats experiencing cocaine withdrawal display anxiogenic-like responding in preclinical models (Hu et al. 2016; de Oliveira Citó Mdo et al. 2012; Kupferschmidt et al. 2012), including the LD emersion test (Costell et al., 1990), providing evidence for this hypothesis. Additionally, this conditioned aversive state is not specific to cocaine, but can be applied to the rewarding drug, morphine.

The experiments in Chapter 3 show that a 30-min delay following a brief (2-min) exposure to LiCl, but not cocaine, produces conditioned gaping reactions. As well, pre-treatments with either ondansetron, SCH 23390 or oleoyl glycine (O1Gly) were able to interfere with the establishment of conditioned gaping to saccharin paired with delayed (10-min) cocaine, but only on early conditioning trials.

Although conditioned gaping is established to saccharin paired with delayed cocaine, this is only true when saccharin is infused intermittently throughout the 30-min delay. When
saccharin infusion precedes the delay, conditioned gaping is only established to LiCl, but not cocaine. This difference was apparent even though both delayed cocaine and delayed LiCl produced an equivalent CTA, a pattern that is present in all our experiments comparing these compounds. Additionally, similar results occur whether the delay occur in the TR chamber or in the home cage. When also taking into consideration our previous experiment where a distinctive context did not elicit conditioned gaping when paired with delayed cocaine (Guenther et al. 2018), it is likely that context is not significantly influencing conditioning in this paradigm.

Again, differing TR profiles between LiCl and cocaine likely indicate different internal states influencing their association with saccharin. LiCl produces nausea and vomiting when administered (Parker 2014), therefore conditioned gaping to saccharin paired with delayed LiCl in this paradigm is likely due to the aversive state of nausea, whereas intermittently presented saccharin paired with delayed cocaine may result in saccharin signaling an aversive negative state over the period during which the rats must wait for administration of cocaine, a rewarding drug.

Various pre-treatments were able to interfere with the establishment of conditioned gaping to saccharin paired with delayed (10-min) cocaine on early conditioning trials (trials 2 and 3), however this effect was overcome with additional conditioning trials for all pre-treatments. Although these results should only serve as a preliminary assessment of the mechanism underlying the establishment of conditioned gaping produced by delayed access to cocaine, they are still worth discussing. Ondansetron is a classic anti-emetic drug that interferes with the establishment LiCl-induced conditioned gaping in rats (Limebeer and Parker 2000). However, 5-HT3 receptor antagonists like ondansetron have also been shown to reduce anxiety-
like behaviour caused by cocaine withdrawal in rats, and potentially reduce cocaine craving (Costall et al. 1989, 1990). SCH 23390 is a dopamine D1 receptor antagonist that has been shown to interfere with the development of addictive behaviours in animal models (Self 2010). However, this compound caused a dramatic reduction in locomotion, therefore results from this group should be interpreted with caution. Finally, OlGly is a newly discovered cannabinoid that can interfere with the establishment of LiCl-induced conditioned gaping (Rock et al. in preparation) and morphine withdrawal by acting on the cannabinoid 1 (CB1) receptor (Petrie et al. 2019).

There are several lines of evidence supporting the contention that conditioned gaping produced by delayed access to cocaine represents an association between a flavour and a conditioned negative affective state that may represent conditioned withdrawal (or the opponent process aversive state) produced by the waiting period for cocaine availability (Wheeler et al. 2008, 2011). The current experiments support this hypothesis by demonstrating the development of aversive TR and increased anxiety with presentation of saccharin associated with delayed reward (cocaine or morphine). Further research should further examine pre-treatments to elucidate the mechanism underlying this phenomenon.
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APPENDIX

Preliminary evaluation of the potential of conditioned gaping elicited by saccharin paired with delayed access to cocaine to modify dopamine release in a microdialysis study
Introduction

It has been established that neurons in the nucleus accumbens (NAc) show differential activity to rewarding and aversive stimuli. Specifically, activity in this brain region can indicate hedonic valence of taste stimuli. Palatable flavours such as sucrose elicit inhibitory responses, whereas unpalatable flavours such as quinine elicit excitatory responses in the NAc (Roitman et al. 2005). In addition, researchers have also examined conditioned responding to cues that predicted flavour administration, with similar activity patterns in the NAc to that of rats acutely exposed to either a palatable or unpalatable flavour (Roitman et al. 2005). A subsequent study examined changes in dopamine (DA) concentrations in the NAc following infusion of either sucrose or quinine using fast-scan cyclic voltammetry (Roitman et al. 2008). Intraoral infusions of the palatable solution sucrose resulted in an increase of DA in the NAc. Contrarily, intraoral infusion of the aversive flavour quinine caused a decreased release of DA relative to baseline (Roitman et al. 2008). Taken together these experiments revealed that the NAc is able to rapidly and accurately discriminate between aversive and rewarding taste stimuli as seen by changes in neuron activity and DA concentrations.

Although these studies allow us to better understand how the NAc responds to the innate palatability of a particular taste, they did not examine how these responses may change if the taste is conditioned to either be devalued or aversive. Wheeler et al. (2008) examined NAc activity in response to a flavour that had been previously paired with delayed cocaine. Like with previous research, when rats are exposed to a palatable flavour prior to conditioning (in this case either orange or grape flavoured saccharin), neuronal activity in the NAc was reduced. However, following the repeated pairings of saccharin and delayed cocaine they observed significant
changes in NAc responding. Specifically, administration of the delayed-cocaine paired saccharin resulted in increased neuronal firing in the NAc, a response similar to that of innately aversive flavours. Results from this study suggest that when saccharin has been paired with delayed cocaine administration it is conditioned to be aversive, a finding that is also supported with behavioural data (Wheeler et al 2008). As for DA concentrations, a similar experiment was conducted and it was found that saccharin that had previously been paired with delayed cocaine elicits decreased DA release in the NAc, which is similar to DA responses to aversive flavours (Wheeler et al. 2011). Together, these studies indicate a shift in hedonic valence of saccharin from palatable to aversive when paired with delayed cocaine, which supports the hypothesis that saccharin can serve as a drug related cue that signals a waiting period for cocaine administration and that this cue may be eliciting a negative affective state as indicated by aversive responding (Wheeler et al. 2008).

Using microdialysis, we examined changes in DA concentrations in the NAc in response to saccharin that had been paired with delayed cocaine administration. Because we had previously observed aversive behavioural responding in animals who received saccharin paired with delayed cocaine, and that prior research has associated aversive flavours with a decrease in DA in the NAc, we expected saccharin that had been paired with delayed cocaine to result in decrease DA concentrations in the NAc.
Methods

Subjects

The subjects were 22 naïve male Sprague-Dawley rats were obtained from Charles River Laboratories (St Constant, Quebec). Rats were between 250-300g in weight on the first conditioning trial. All rats were individually housed in home cages made of opaque white plastic (48 × 26 × 20 cm), containing bed-o-cob bedding from Harlan Laboratories, Inc. (Mississauga, Ontario), a brown paper towel, and Crink-l'Nest™ from The Andersons, Inc. (Maumee, Ohio). All rats were subjected to an ambient temperature of 21°C and a 12/12 hour light-dark schedule (lights off at 7 AM), such that behavioural testing was conducted during the dark phase of the light cycle. Rats were maintained on food (Highland Rat Chow) and water ad-libitum.

Surgery

All rats were implanted with a unilateral indwelling guide cannula into the NAc while under isoflurane anesthesia, which would allow the microdialysis probe to be inserted during the experiment. Stereotaxic surgery followed the procedure described by Limebeer et al (2018). A 21-gauge guide cannula was set at a convergent 10° angle at +1.6 mm AP, +3.2 mm ML and -6.6 DV from bregma. Half of the rats were cannulated in the right hemisphere and half in the left hemisphere. Following removal from the stereotaxic frame rats were implanted with an intraoral (IO) cannula as described in Limebeer et al (2010). Rats underwent 5 days of post-surgical monitoring. Following completion of the experiment, histology was done to verify guide cannula placements (histology procedure described in Limbeer et al. 2018).
Drugs

Cocaine was mixed at a concentration of 1.5 mg/ml in solution with saline (SAL) for subcutaneous injection in order to prevent skin necrosis. As well, the injection site was changed on a daily basis. A dose of 20 mg/kg cocaine was used and was administered at 13.3 ml/kg.

Apparatus

For conditioning trials, rats were placed in a clear Plexiglas box (22.5 x 26 x 20 cm) with an opaque lid, that sits on top of a clear glass topped table. The chamber was located in a dark room next to a 25W light source. Test trial occurred in a separate microdialysis chamber. This chamber was made of a grey Plexiglas box (60 x 40 x 40 cm) with bed-o-cob bedding on a plastic floor. This plastic floor could be removed, resulting in the chamber sitting on a clear class table with a mirror located under the chamber at a 45° angle in order to allow viewing of the rats ventral surface. This plastic floor was removed during saccharin infusion to allow recording of orofacial responses. There was a video camera (Sony DCR-HC48, Henry’s Cameras, Waterloo, ON, Canada) placed in front of the mirror to allow the trials to be recorded and scored at a later time. Trials were scored by an observer blind to the experimental groups using ‘The Observer’ (Noldus Information Technology Inc., Leesburg, VA, USA).

Procedure

All rats were adapted to the taste reactivity (TR) procedure three days prior to the first conditioning trial. During adaptation, the rat was placed in the TR chamber, their cannula attached to an infusion pump (Model KDS100, KD Scientific, Holliston, MA, USA) for fluid
delivery. During the adaptation trial, each rat was intraorally infused with reverse osmosis water for 10 seconds every minute for 20 minutes at a rate of 1.2 ml/min, for a total delivery of 4 ml of water over the course of the 20 minutes.

Three days following adaptation, the first conditioning trial began. On each of 4 conditioning trials, the rat was placed in the TR chamber, its cannula was attached to the pump for the infusion of 0.1% saccharin, infused according to the same schedule as water during adaptation. After the 20-minute conditioning trial, the rat was removed from the chamber and given an immediate injection of VEH (n=11) or cocaine (20 mg/kg sc (n=11). Following the injection, the rat was placed back in its home cage. The test trial occurred in the microdialysis chamber 24 hrs after the final conditioning trial.

At the beginning of test day, a microdialysis probe was inserted into the guide cannula of the rat which allowed for the perfusion of artificial cerebrospinal fluid (aCSF: NaCl 147 mM, KCl 2.8 mM, CaCl₂ 1.2 mM, MgCl₂ 1.2 mM; pH 7.4) from the NAc at a rate of 0.6 μl/min. The microdialysis probes consist of 2.5 mm length of semipermeable dialysis membrane from Spectra/Por in-vivo Microdialysis Hollow Fibers, 2 μm OD, 13,000 MW cut off. A 120-minute acclimatization period followed insertion of the probe where samples were not collected. Baseline sample collection commenced after acclimatization, where samples were collected for 20 minutes each, then immediately frozen. Three baseline samples were taken over the course of 60 minutes. Following collection of baseline samples, intraoral cannula were attached to an infusion pump and rats were infused with saccharin, as in the conditioning procedure (20-min), while a dialysate sample was collected. Following termination of the saccharin infusions, post-
Infusion samples were collected at 20-minute intervals, for a total of 80 minutes (4 post-infusion samples).

**HPLC detection of DA**

The dialysate from the samples were analyzed for DA using the Eicom HTEC-510 HPLC/ECD system (Eicom USA). Each sample was extracted from the vial and loaded on a C-18 reverse-phase column (PP-ODS II, 4.6 x 30 mm, Eicom USA) using a manual injection port (Rheodyne 9725i; 20 µl loop). The column was maintained at a temperature of 25°C with a mobile phase (0.1 M Phosphate buffer pH5.4 including 1.5% methanol, 500 mg/L Decansulfonate sodium salt [DSS] and 50 mg/L 2Na-EDTA) set at a flow rate of 0.6 ul/min. Electrochemical detection of DA was determined using a graphite working electrode (WE-3G, Eicom USA) maintained at a potential of +450 mV relative to an Ag/AgCl reference electrode (RE-500, Eicom USA).

**Experimental Design and Statistics**

The concentration of DA in the dialysate samples were converted to percent baseline, determined by the mean pg/µl of the 3 baseline readings prior to intraoral saccharin infusion. The mean percent baseline measures of DA (pg/µl) were entered into mixed factor ANOVAs with the between group factor of conditioning group (Sac->Saline, Sac->Cocaine) and the within group factor of time of sample (a total of 8). Statistical significance was defined as p < 0.05.
Results

Taste reactivity

The aversive behaviour of gaping was entered into a 2 x 4 mixed factors ANOVA, where the 20-minute test trial was separated into 5-minute time intervals. There was a significant effect of group, $F(1, 20) = 8.6, p = 0.008$, with the cocaine (20 mg/kg) group exhibiting significantly more gaping than VEH, as seen in Figure 1.

Microdialysis

There were no significant differences in DA release between the two conditioning groups, but the levels of DA increased across the first 100 min of testing, including during the Sac infusion (for both groups). Figure 1 presents the mean ($\pm$sem) percent baseline of DA (pg/µl) during each period of sampling. The 2 x 8 mixed factor ANOVA revealed only a significant effect of time, $F(7, 140) = 3.7; p = 0.001$; there was a significant linear trend across minutes ($p < 0.01$). Paired tests between consecutive minutes revealed a significant increase in DA in the NAcc between min -40 and -20 ($p < 0.01$) and min 0 and plus 20 during the saccharin infusion ($p < 0.25$), but a reduction in DA between min 40 and 60 ($p < 0.01$) that remained low. Results are depicted in Figure 2.

Discussion

It was expected that if the saccharin paired with delayed cocaine had been conditioned to be aversive, then a decrease in DA would be seen in the NAc when rats were re-exposed to the saccharin. However, percent baseline DA levels during the Saccharin infusion or afterwards in
group Sac → cocaine did not significantly differ from that of group Sac → saline. Instead, an infusion of saccharin produced an increase in DA compared to baseline in both groups. This indicates an effect of saccharin administration in both groups, but no conditioning effect of decreased DA to delayed-cocaine paired-saccharin. Although this finding would suggest that the saccharin has not been conditioned to be aversive (Roitman et al. 2005, 2008), the behavioural data contradicts this conclusion. Rats who received saccharin paired with delayed cocaine also exhibited the aversive reaction of gaping despite not showing decreased DA in the NAc. It is possible that the temporal resolution (20 min samples) prevented an assessment of changes in DA in response to the Sac-paired cocaine; that is, the initial reaction to cocaine may have been suppressed DA which washed out over the 20 min sampling period. Unfortunately, this potential problem cannot be resolved using microdialysis techniques.
Mean (± SEM) frequency of gaping with 20-min delayed cocaine (20 mg/kg sc). Asterisks indicate a group difference from saline; *p < 0.05, **p < 0.01, ***p < 0.001.
Mean (± SEM) % baseline DA in the NAc. Samples were collected every 20 minutes for a total of 160 minutes. Rats were intraorally infused with saccharin for 10 seconds every minute for 20 minutes, starting at time 0.