Intracerebroventricular (ICV) Administration of a CB₁ Inverse Agonist as a Potential Model with which to Examine the Influence of the Endocannabinoid System in Anhedonia

by

Danielle A. L. Haynes

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ABSTRACT

INTRACEREBROVENTRICULAR (ICV) ADMINISTRATION OF A CB₁ INVERSE AGONIST AS A POTENTIAL MODEL WITH WHICH TO EXAMINE THE INFLUENCE OF THE ENDOCANNABINOID SYSTEM IN ANHEDONIA

Danielle A. L. Haynes

University of Guelph, 2016

Advisors: Professors Francesco Leri and Linda A. Parker

Recent evidence has implicated the CB₁ receptor in anhedonia. CB₁ inverse agonists in particular have been shown to elicit anhedonia in humans, and suppress intake and hedonic response to palatable food in laboratory animals, indicative of anhedonia. However, the majority of these findings in animals originate from studies employing systemic administration, which also results in nausea. Recent evidence indicates administration of CB₁ inverse agonists directly to the lateral ventricles eliminates the nausea component. The present thesis examined whether chronic and/or acute intracerebroventricular (ICV) administration of CB₁ inverse agonist AM251 can induce anhedonia. Hedonic capacity was assessed via ad libitum consumption of a high-fructose corn syrup (HFCS) solution and taste reactivity to HFCS. Chronic, but not acute, ICV AM251 resulted in a transient, selective decrease in HFCS consumption, suggesting that chronic central CB₁ inverse agonism can induce an acute anhedonic state, and further implicating the central CB₁ receptor in clinical anhedonia.
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Finally, I would like to dedicate this thesis to my dog, Sasha, who passed away while I was writing it. You were the greatest companion and friend anyone could ask for. I love and miss you dearly.
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LIST OF ABBREVIATIONS

2-AG..................................................................................................................... 2-arachidonoylglycerol
aCSF.......................................................................................................................... artificial cerebrospinal fluid
cAMP.......................................................................................................................... cyclic AMP
CB₁............................................................................................................................ cannabinoid receptor type 1
CB₂............................................................................................................................ cannabinoid receptor type 2
DA............................................................................................................................ dopamine/dopaminergic
DMSO.......................................................................................................................... dimethyl sulfoxide
FAAH......................................................................................................................... fatty acid amide hydrolase
GABA......................................................................................................................... γ-aminobuteric acid
HFCS......................................................................................................................... high fructose corn syrup
i.p. ............................................................................................................................... intraperitoneal
ICV............................................................................................................................. intracerebroventricular
MAGL......................................................................................................................... monoacylglycerol lipase
MDD........................................................................................................................... major depressive disorder
NAc............................................................................................................................. nucleus accumbens
PEG400..................................................................................................................... polyethylene glycol 400
PFC............................................................................................................................. prefrontal cortex
s.c. ............................................................................................................................. subcutaneous
TR............................................................................................................................... taste reactivity
VP............................................................................................................................... ventral pallidum
VTA............................................................................................................................. ventral tegmental area
Δ9-THC...................................................................................................................... Δ9-tetrahydrocannabinol
GENERAL INTRODUCTION

Brief Rationale & Summary of Experiments

Anhedonia, commonly defined as the inability to experience pleasure, is a prominent symptom of major depressive disorder (MDD) and schizophrenia. Recent evidence suggests that anhedonia is moderated by several different systems, which are each responsible for specific reward processing-related deficits, including deficits in reward anticipation, reward-seeking motivation, and hedonic sensitivity to reward. The endocannabinoid system, particularly the centrally-abundant CB$_1$ receptor, has been implicated in hedonic processing: exogenous CB$_1$ agonists are associated with increases in hedonic response to highly-palatable foods in laboratory animals, while CB$_1$ inverse agonists, which induce opposing behavioural and neurological effects, have been shown to elicit typical anhedonic pathology, suppressing palatable food consumption, hedonic response to highly palatable flavours, and operant responding for a palatable food reward. However, the majority of these findings with respect to inverse agonists originate from studies employing systemic administration, which has been shown to elicit nausea. As anhedonia in animals is commonly assessed via changes in response to palatable food, nausea presents a significant potential confound. Recent evidence indicates that CB$_1$ inverse agonists administered directly to the lateral ventricles eliminates the nausea component, while preserving hedonic response to palatable flavours. Consequently, intracerebroventricular (ICV) administration appears to be a promising route for examining the role of the endocannabinoid system in anhedonia.

The present study sought to establish whether chronic central administration of a CB$_1$ inverse agonist (AM251) is capable of inducing a persistent anhedonic state independent of any
peripheral effects, particularly nausea. Anhedonia was assessed via palatable food intake versus regular chow and water, as well as via hedonic taste reactivity (TR) to a palatable flavour.

Anhedonia

Anhedonia, or the inability to experience pleasure, is a prominent symptom of MDD, as well as some forms of schizophrenia. Anhedonia in humans has typically been characterized as a diminished interest in pleasurable activities: studies of anhedonic individuals have revealed frequent self-reported disinterest in otherwise pleasurable experiences or stimuli, as well as a decreased propensity to seek out or to expend effort to obtain a reward (McFarland & Klein, 2009; Sherdell, Waugh, & Gotlib, 2012; Yang et al., 2014) and, in the case of schizophrenia, reduced anticipatory response to reward (Gard, Gard, Kring, & John, 2006).

While human studies are able to assess response to a variety of different rewards, animal studies examining anhedonia are necessarily more limited. In order to assess anhedonia in laboratory animals, studies frequently use changes in response to a highly palatable food as a measure of hedonic capacity. Within these studies, a reduction in highly palatable food intake, operant responding for a highly palatable food reward, and/or hedonic reactivity to a palatable flavour are considered to be indicative of an anhedonic state (Muscat & Willner, 1992; Peciña, Berridge, & Parker, 1997; Willner, Moreau, Kurre Nielsen, Papp, & Sluzewska, 1996; Wilmouth & Spear, 2009).

Recent clinical and behavioural research has identified several different reward processing-related deficits, all of which can contribute to the expression of an anhedonic endophenotype (reviewed by Der-Avakian & Markou, 2012). Anticipatory deficits, characterized by the inability to develop and recognize meaningful associations between contextual cues and
rewards, are associated with a number of learning and reward-related regions, including the nucleus accumbens (NAc), ventral pallidum (VP), hippocampus, and caudate nucleus (Tsurugizawa, Uematsu, Uneyama, & Torii, 2012), as well as striatal dopaminergic (DA) activity (Wang, Volkow, & Fowler, 2002; as cited by Der-Avakian & Markou, 2012). Deficits in motivation to seek out and/or work for a reward have been associated with the mesolimbic DA pathway (Berridge & Kringelbach, 2008; Berridge & Robinson, 1998; Kelley et al., 2002; Salamone, Correa, Mingote, & Weber, 2003). Finally, deficits in hedonic sensitivity, in which an individual derives subnormal pleasure from a reward, are associated with the ventral striatum, as well as μ-opioid, GABA, and endocannabinoid receptors (Faure, Richard, & Berridge, 2010; Kelley et al., 2002; Sanchis-Segura, Cline, Marsicano, Lutz, & Spanagel, 2004).

The Endocannabinoid System

The endocannabinoid system is comprised of two G protein-coupled receptors, cannabinoid receptor type 1 (CB₁) and type 2 (CB₂), as well as their endogenous ligands and corresponding degradational enzymes. While recent evidence suggests that CB₂ receptors are indeed present within the central nervous system (Pacher & Mechoulam, 2011; Van Sickle, 2005), they have not yet been found to have any direct psychoactive effects. Rather, it is the centrally-abundant CB₁ receptor which is the primary receptor involved in endocannabinoid-mediated psychological mechanisms.

Central CB₁ receptors are located on the presynaptic terminals of a variety of different neurons, including both GABAergic and glutamatergic neurons (Hill et al., 2007). CB₁ receptors possess two primary endogenous ligands, N-arachidonylethanolamine (anandamide) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995),
which are rapidly degraded by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Giang & Cravatt, 1997) and monoacylglycerol lipase (MAGL) (Dinh et al., 2002). These compounds are synthesized and released on demand by the postsynaptic cell into the synaptic cleft, where they bind to and activate the CB1 receptors on the presynaptic neuron. Activation of these receptors inhibits N-type calcium channels, reducing intracellular calcium levels, while simultaneously activating K+ channels (Guo & Ikeda, 2004; Lauckner, Hille, & Mackie, 2005; Mackie & Hille, 1992). CB1 activation likewise inhibits adenylyl cyclase (Howlett, 1985), resulting in reduced intracellular cyclic AMP (cAMP) (Wade, Tzavara, & Nomikos, 2004). Through these mechanisms, CB1 receptor activation serves to inhibit subsequent neurotransmitter release within the presynaptic cell. Consequently, the CB1 receptor appears to play a key role in modulating synaptic transmission throughout the central nervous system.

**Cannabinoids and Reward**

The endocannabinoid system has been implicated in reward processing and hedonic sensitivity to reward (Der-Avakian & Markou, 2012; Gardner, 2005). The exogenous CB1 agonist Δ9-tetrahydrocannabinol (Δ9-THC), the primary psychoactive constituent of *Cannabis sativa*, has been well-established to induce feelings of euphoria in humans. Further, while high doses of Δ9-THC are generally aversive in lab animals, low doses of Δ9-THC (.075 - .75 mg/kg i.p.), as well as administration of Δ9-THC directly to the posterior VTA and the posterior shell of the NAc, are capable of inducing a place-preference, and animals will readily self-administer Δ9-THC to these areas (Robinson, Hinder, Pertwee, & Riedel, 2003; Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006).
WIN55,212-2, another CB₁ agonist, has been shown to attenuate chronic mild stress-induced depressive symptomatology, including anhedonia (Segev, Rubin, Abush, Richter-Levin, & Akirav, 2013). Likewise, genetic deletion of the CB₁ receptor results in an anhedonic phenotype: Sanchis-Segura and colleagues (2004) found that CB₁ knockout mice consumed less sucrose than their wild-type equivalents within both operant self-administration and a two-bottle test paradigm. When sucrose was devaluated by adding bitter quinine to the solution, this disparity in consumption between knockout and wildtype mice was eliminated. These findings combined present a robust body of evidence to suggest that the CB₁ receptor is heavily implicated in reward sensitivity and processing, particularly within mesolimbic DA structures.

Cannabinoids and Anhedonia

While exogenous CB₁ agonists tend to enhance reward response, another subtype of exogenous cannabinoids, known as inverse agonists, has been shown to elicit behavioural and neurological effects contrary to those produced by agonists. Prominent inverse agonist rimonabant (trade name Acomplia®) initially showed promise as an anti-obesity treatment due to its capacity to curb appetite and promote weight loss (Després, Golay, & Sjöström, 2005; Van Gaal, Rissanen, Scheen, Ziegler, & Rössner, 2005). After passing clinical trials, rimonabant was briefly available in Europe and South America as a weight-loss drug; however, it was quickly removed from the market in light of mounting evidence of serious depressive side effects, including heightened anxiety and suicidality (reviewed by Christensen, Kristensen, Bartels, Bliddal, & Astrup, 2007; Després et al., 2005). Inverse agonists have since garnered interest as a potential window of insight into the role of the endocannabinoid system in the development of depressive symptoms, including anhedonia.
From a neurological perspective, CB₁ inverse agonists appear to behave in a manner directly opposite to that of agonists. Inverse agonists antagonize the receptor, preventing endogenous agonists from binding, while simultaneously exerting their own neurochemical effects (reviewed by Pertwee, 2005). While agonists decrease intracellular calcium levels, thereby suppressing neurotransmitter release (Mackie & Hille, 1992), inverse agonists are associated with an increase in intracellular calcium levels (Pan, Ikeda, & Lewis, 1998). CB₁ inverse agonism has also been associated with increased available cAMP (Mato, Pazos, & Valdizán, 2002). Consequently, it appears that inverse agonists promote, rather than inhibit, neurotransmitter release, thereby eliciting neurological effects directly inverse to that of normal function.

CB₁ inverse agonists have also been shown to induce an anhedonic state similar to that observed in MDD and schizophrenia: rimonabant reduced neural activation in the right ventral striatum and putamen, as well as the mid-orbitofrontal cortex, in response to a pleasurable stimulus in healthy adults (Horder, Harmer, Cowen, & McCabe, 2010). This compound likewise reduced activation in the caudate and left ventral striatum, and enhanced activation in the lateral orbitofrontal cortex in response to an aversive stimulus within the same participants. This pattern of activation is similar to those observed in patients experiencing anhedonia (Epstein et al., 2006; Horder et al., 2010; Keedwell, Andrew, Williams, Brammer, & Phillips, 2005)

Inverse agonists have also been shown to attenuate the rewarding and reinforcing effects of cocaine, heroin, alcohol, and nicotine (Cohen, Perrault, Voltz, Steinberg, & Soubrié, 2002; Colombo, Vacca, Serra, Carai, & Gessa, 2004; De Vries, Homberg, Binnekade, Raasø, & Schoffelmeer, 2003; Navarro et al., 2001), as well as reduce intracranial self-stimulation of the
median forebrain bundle within animal models (Deroche-Gamone, Le Moal, Piazza, & Soubrié, 2001). Further, inverse agonists interfere with highly palatable food-reinforced conditioned behaviour (Droste, Saland, Schlitter, & Rodefer, 2010; Freedland, Sharpe, Samson, & Porrino, 2001; Ward & Dykstra, 2005), as well as selectively yield reductions in palatable food consumption (Beyer et al., 2010; Higgs, Williams, & Kirkham, 2003; Miner et al., 2008) and hedonic reactions to highly-palatable sweet flavours (Jarrett, Scantlebury, & Parker, 2007), all of which are suggestive of an anhedonic state. Consequently, there is considerable evidence to suggest that the endocannabinoid system may be implicated in the development of clinical anhedonia.

However, a majority of the evidence to support the involvement of the endocannabinoid system in anhedonia originates from studies in which an inverse agonist was administered systemically, which has been shown to elicit nausea in humans (Després et al., 2005; Van Gaal et al., 2005) and in animals (Limebeer et al., 2010; McLaughlin et al., 2005). As anhedonia in animal models is frequently assessed via changes in palatable food consumption, palatable food-reinforced conditioned behaviour, and hedonic reactivity to palatable flavours, any change in these measures as a result of CB₁ inverse agonist administration may be at least partially the result of nausea, as opposed to anhedonia. Recently, it was determined that administration of a CB₁ inverse agonist directly to the lateral ventricles of the brain bypasses the nausea component while still reducing hedonic taste reactivity to a highly-palatable saccharin solution (Limebeer et al., 2010). Consequently, the lateral ventricles represent a promising potential route for examining the role of the endocannabinoid system in anhedonia.
**Taste Reactivity**

Taste reactivity (TR) is a behavioural assessment of hedonic response to a particular flavour first devised by Grill and Norgren (1978). In this paradigm, a tastant, such as saccharin, sucrose, or quinine, is infused directly into the oral cavity of a freely-moving animal. The animal’s orofacial response to the tastant is recorded and quantified. Positive hedonic response in rats is characterized by repeated protrusion of the tongue from the oral cavity, while negative or aversive hedonic response is characterized by a gaping reaction, in which the mouth opens widely in a triangular fashion and the lower incisors are clearly visible (Grill & Norgren, 1978). Endocannabinoid agonists and antagonists have been found to influence hedonic taste response: low doses of CB₁ agonist Δ9-THC enhance sucrose palatability and reduce the aversiveness of quinine; further, systemic and ICV administration of CB₁ inverse agonist AM251 has been found to suppress sucrose and saccharin palatability within this paradigm (Jarrett, Limebeer, & Parker, 2005; Jarrett et al., 2007; Limebeer et al., 2010). As a result, taste reactivity appears to be a prime candidate for assessing any potential link between central CB₁ inverse agonism and reduction in hedonic capacity.

**High-Fructose Corn Syrup**

High-fructose corn syrup (HFCS) is a caloric sweetener derived from corn which has increasingly come to replace sucrose within Western diets. The most common formulation, HFCS-55, is composed of 42% glucose and 55% fructose, as well as 3% glucose polymers. Sucrose, conversely, is a disaccharide composed of 50% glucose and 50% sucrose. Like sucrose, HFCS has likewise been shown to be highly rewarding: rats will readily self-administer intra-oral infusions of 8%, 25%, and 50% HFCS in water, with 50% yielding the greatest number of operant responses and highest breakpoint on a progressive-ratio schedule (Levy et al., 2014).
While responding on a fixed-ratio schedule is maintained for longer by sucrose than by HFCS at isocaloric concentrations, HFCS has been shown to be more potent than sucrose, maintaining a similar level of responding for intra-oral infusion at a lower concentration (25% HFCS vs. 32% sucrose). Additionally, there is some evidence to suggest that the 3% glucose polymers, though not consciously discernable in humans, contribute to its reinforcing quality: rats exhibit a greater preference for sucrose and HFCS than an isocaloric mixture of 55% fructose and 45% glucose (Ackroff & Sclafani, 2011). Given its increasing abundance in processed food and robust reinforcing effects, HFCS appears promising as another potential palatable fluid with which to assess endocannabinoid-mediated anhedonia.
EXPERIMENT 1

Introduction

Experiment 1 was designed with the intention of assessing whether chronic ICV AM251 is capable of producing a persistent anhedonic state within laboratory rats. AM251 or vehicle was administered continuously to the right lateral ventricle via subcutaneous osmotic minipump using doses selected from Limebeer et al. (2010). The sham group received sham implantation surgery. Due to chronic nature of the present study, the vehicle utilized consisted of a 1:1 DMSO:PEG400 solution, shown to be an effective vehicle for AM251 for up to 21 days within a subcutaneous minipump by Rahn et al. (2014). Hedonic capacity was assessed in two ways: *ad lib* in-cage consumption of a highly palatable fluid (50% HFCS) versus regular chow and water intake, and TR to an intraoral infusion of 50% HFCS. If chronic ICV AM251 is indeed capable of inducing anhedonia, it was expected to manifest as a selective reduction in HFCS intake, as well as reduced TR to intraoral HFCS infusion. For a timeline of the procedures utilized in Experiment 1, see Figure 1.

Methods

Drugs and Solutions

Low (1.25 μg/day; 0.052 μg/h), medium (12.5 μg/day; 0.52 μg/h), and high (300 μg/day; 12.5 μg/h) doses of inverse agonist AM251 were dissolved in a 1:1 solution of DMSO:PEG400. Vehicle consisted of only 1:1 DMSO:PEG400.
For the angiotensin test, angiotensin II was prepared at a concentration of 25 μg/mL and dissolved in physiological saline. High fructose corn syrup (HFCS) was prepared at a concentration of 50% in combination with reverse-osmosis water.

Subjects

Forty-two experimentally-naive male Sprague-Dawley rats initially weighing 225-250 grams were used in this experiment (Charles River Laboratories, St Constant, Quebec). Animals were single-housed at a constant temperature of 21°C on a reverse light:dark schedule (lights off at 0700 h) with ad libitum access to standard rodent chow and water. All experimental manipulations were conducted during the dark cycle. All procedures in this study were approved by the University of Guelph Animal Care Committee, and all animals were handled and housed in accordance with the policies outlined by the Canadian Council on Animal Care.

Surgical Procedures

Intracerebroventricular Cannulation

Animals were first anaesthetized with 5% isoflurane gas mixed with pure oxygen. Once anaesthetized, animals were administered an anti-inflammatory drug (Carprofen, 0.1 ml/kg i.p.) and a topical anaesthetic (Marcaine/Lidocaine solution, 0.1 ml s.c. on either side of incision site). A small strip of skin beginning between the ears and terminating between the eyes was shaved, and the area was disinfected with antibacterial soap, 70% isopropanol, and 30% betadine. Animals were then secured in the stereotaxic frame and an incision was made along the dorsal surface of the skull. Connective tissue was cleared away with swabs and the incision was held open by four haemostats to expand the surgical field. A small hole was made in the skull with a handheld drill and a 90° bend intracranial cannula (ALZET, Durect Corporation, California) was
implanted unilaterally in the right lateral ventricle (-2.0 mm ML, -1.0 AP, -4.0 DV from Bregma). The exterior port of each cannula was aligned to face the posterior. Due to the angular nature of the cannula, it was not possible to insert an obturator to maintain patency. Consequently, the cannula was flushed with artificial cerebrospinal fluid (aCSF) prior to implantation to prevent tissue blockage and, once the cannula was in position, 1.0 μl of aCSF was microinfused over 1 minute through the cannula into the right lateral ventricle to confirm the absence of any blockage and to promote patency. Each cannula was then secured in place by a dental cement head cap formed around four small screws anchored in the skull. Each cannula was then capped with a small length of Polyethylene microinfusion tubing sealed at one end with a soldering iron, to prevent potential blockages by foreign material.

**Intra-Oral Cannulation**

Once the intracranial cannula was secured to the skull, the animal was removed from the stereotaxic frame and an intraoral cannula was implanted as described by Levy et al. (2014) and Limebeer et al. (2010). A small strip of skin at the base of the neck was shaved and disinfected. An additional 0.1 ml of Marcaine/Lidocaine solution was administered s.c. at this site. Topical Lidocaine was applied using a swab to the inside of the cheek on the side to be cannulated. A 15 gauge stainless steel guide cannula was then inserted at the base of the neck and was guided subcutaneously below the ear until it the tip rested along the inside of the cheek behind the first molar. The tongue was moved away using forceps, and the blunt end of the forceps was inserted into the mouth of the animal. The tip of the guide cannula was pushed through the cheek and guided out of the mouth along the blunt end of the forceps. An intraoral cannula, approximately 100 – 130 mm in length made from PE90 polyethylene tubing with one flanged end was inserted into the guide cannula. The guide cannula was then removed, leaving the intraoral cannula in
place. A small surgical mesh disc (approx 10 mm diameter) was threaded onto the exterior portion of the intraoral cannula and coated with betadine, and the cannula was secured with three small squares of elastic. Finally, the cannula was flushed with 2-3 ml of an antibacterial oral rinse. Animals were allowed 5 days of recovery prior to any experimental manipulations.

Osmotic Minipump Implantation

Subcutaneous osmotic minipumps (ALZET, Durect Corporation, California) were filled with either low, medium, or high dose AM251, or vehicle solution, and a short length of PE50 polyethylene tubing was attached to the port of the pump. Pumps were primed in 37°C physiological saline overnight prior to the surgery.

During implantation surgery, animals were first anaesthetized as previously described, and were administered 0.1 ml/kg Carprofen \textit{i.p.} and 0.1 ml Marcaine/Lidocaine solution \textit{s.c.} at the site of incision. A small strip of skin between the scapulae was shaved and disinfected. An incision at this site was made with a scalpel, and subcutaneous pocket was formed by inserting a large haemostat between the skin and muscle tissue. The osmotic pump was inserted in this pocket. The skin at the posterior base of the head cap was gently separated from the dental cement to form a small hole through which to thread the tubing. The distal end of the tubing was clamped using another smaller haemostat and was threaded subcutaneously from the initial incision through the small hole. The polyethylene cap was removed from the port of the intracranial cannula. The minipump tubing was cut to an appropriate length such that the head of the animal could move unrestricted while the pump sat between the shoulder blades. The tubing was then secured to the exterior port of the cannula using ethyl acrylate, and the incision was closed with wound clips. Animals in the sham group underwent the same surgical procedure,
with the exception that no pump was implanted, and the polyethylene cap remained on the intracranial cannula. Due to the immediate onset of administration via the subcutaneous pump, experimental manipulations began the following day.

**Experimental Procedures**

**Angiotensin Test**

An angiotensin test was conducted in order to confirm placement and patency of the intraoral cannula. Animals were microinfused with 1-2 µl of angiotensin II and returned to their home cages and observed. Animals which drank water within the first minute post-infusion, drank continuously for at least one minute within the first five minutes, and consumed in excess of 2 ml of water within a 30-minute window were considered to have correctly-placed and patent cannulas. Any animal which failed the angiotensin test was assigned to the sham group.

**In-Cage Consumption**

In-cage consumption measurements began at 0700h on the sixth day post-cannulation surgery. Animals were given free, 24h access to a 50% HFCS solution via an additional drinking tube. Animals had *ad libitum* access to water and standard laboratory chow. Food, water, and HFCS consumption measurements were taken at 0700h (lights off), 0800h, 1300h, and 1900h (lights on) in order to probe for any changes in natural circadian feeding patterns. Baseline measurements were recorded for a total of 13 days. Animals were then assigned to one of the five treatment groups: low, medium, or high-dose AM251, vehicle, or sham. Groups were counterbalanced by weight, HFCS intake over the last 3 days of baseline consumption, baseline hedonic response (as measured via TR, described in the following section).
Animals were implanted with a subcutaneous osmotic minipump, or underwent sham surgery on day 14 of HFCS exposure. Food, water, and HFCS consumption were measured for an additional 10 days in the same manner as during baseline. Pumps were then removed and dissected to confirm drug administration had taken place. Following the experiment, animals were humanely euthanized and blood and brain samples were taken for later mRNA analysis (not presented).

*Taste Reactivity*

Taste reactivity was conducted in the manner described by Limebeer et al. (2010). On day 10 of the baseline consumption period, animals underwent a habituation session in which four animals were placed simultaneously in 4 adjacent clear Plexiglas chambers, each with a black Plexiglas lid, situated on a glass stage. A 45° angled mirror was fixed below the glass stage to allow for observation of the ventral side of the animal. Prior to being placed in the chamber, each animal’s intraoral cannula was cleared with a short infusion of air. Each animal’s intraoral cannula was then attached to an infusion line and 1.5 ml of water was infused directly into their mouths over a period of 3 minutes. Immediately following the session, the cannula was flushed with approximately 3 ml of water. The following day, each animal underwent a test trial, during which they were placed one at a time in a singular clear Plexiglas chamber, and 1.5 ml of 50% HFCS was infused directly into their mouth over 3 minutes at a rate of 0.5 ml/min. Hedonic facial response to the infusion was captured using a digital video camera. This test trial was repeated 2 days post-pump implantation, and again 11 days post-implantation, to probe for changes in hedonic response as a result of chronic AM251 administration. Tongue protrusions in response to the intraoral infusion were later manually quantified using Noldus *Observer XT* observation software.
Statistical Analysis

Unadjusted intake, weight, and TR analyses across drug administration days, as well as HFCS intake across habituation days, were analyzed via two-way repeated measures ANOVA. Intake analyses within days and at specific time intervals within a given day were analyzed via one-way ANOVA. All post-hoc analyses were conducted using Fischer’s LSD. Adjusted intake and TR data for each drug day were obtained by dividing daily intake by intake on the final baseline day prior to minipump implantation and multiplying by 100 to obtain a “percentage of baseline” value (referred to hereafter as “baseline-adjusted”), and were analyzed via one-way ANOVA. Negative intake values were attributed to measurement error and were removed from the data set prior to analysis. Nonsignificant statistics are not reported. Analyses were conducted using SigmaStat version 12.2 and SPSS version 20.0.

Results

Angiotensin Test

A total of 17 of the 42 animals used in this experiment did not fit the criteria as having passed the angiotensin test. As their cannula placement and patency could not be confirmed, these animals were assigned to the sham group.

Combining Controls

No significant differences were found between controls (vehicle and sham) with respect to any of the metrics assessed in this experiment (body weight, unadjusted HFCS, water, and food intake, baseline-adjusted HFCS, water, or food intake, and taste reactivity). Consequently, the vehicle and sham groups were combined to form a single “control” group for subsequent analysis.
**Body Weight**

The ANOVA revealed a main effect of drug day \( [F(12,443) = 244.027, p < 0.001] \), as well as a significant interaction between drug condition and day \( [F(48,443) = .2682, p < 0.001] \) (figure not presented). However, no main effect of drug condition was found, nor did the interaction yield any significant post-hocs.

**In-Cage Consumption**

*Unadjusted Intake*

The ANOVA revealed that while there was no main effect of drug condition on unadjusted HFCS intake, nor an interaction, there was a significant main effect of day, \( [F(9,340) = 8.939, p < 0.001] \). Analysis of water intake indicated no significant effect of drug condition, nor was there an interaction between drug condition and day (figure not presented). However, there was a significant main effect of day \( [F(9,340) = 4.127, p < 0.001] \). Likewise, there was a significant main effect of day with respect to food intake \( [F(9,339) = 5.354, p < 0.001] \); however, no significant main effect of drug condition was found, nor was there an interaction.

*Baseline-Adjusted Intake*

While no significant drug effect was found with respect to unadjusted intake, these analyses did not take baseline (pre-surgery) intake into account. Consequently, while intake between groups on a given drug day may not differ significantly, the degree of difference in intake between that particular day and baseline may indeed differ between groups. In order to examine this possibility, unadjusted intake data was converted into a percentage-of-baseline intake (hereafter referred to as “baseline-adjusted”) and additional analysis was performed on this
transformed data set. As a result of this transformation, it was necessary that data within each
day was analyzed independently via a series of one-way ANOVAs.

Baseline-adjusted HFCS intake data is presented in Figure 1. Analysis revealed a
significant effect of drug condition on Day 1 \( F(3,38) = 3.619, p = 0.022 \). Post-hocs revealed
that animals receiving the highest dose of AM251 consumed significantly less HFCS than
animals receiving the medium dose \( p = 0.004 \), or the control group \( p = 0.021 \) versus baseline.
No other days were found to differ significantly with respect to HFCS intake.

Adjusted water and food intake are presented in Figures 2 and 3. Neither food nor water
intake differed significantly between groups on the first day of drug administration, or on any
other day. These results combined indicate that the highest dose of chronic ICV AM251
selectively reduced highly palatable food consumption on day 1 of drug administration,
suggesting the induction of an acute anhedonic state.

*Day 1 Intake*

Day 1 HFCS intake broken down by time of day can be seen in Figure 4. Results revealed
that within the first 12 hours of administration, there was a significant effect of drug condition on
HFCS intake \( F(3,38) = 4.945, p = 0.005 \). Post-hocs indicated that HFCS intake in animals
receiving the highest dose of AM251 was significantly lower than the control \( p = .002 \), low-
dose \( p = .002 \), and medium-dose \( p = 0.13 \) groups.

No significant difference in HFCS intake was found after the first 12 hours. Further, food
and water intake, presented in Figures 5 and 6, did not differ between groups at any time point
during the first day of drug administration. These results combined suggest that the highest dose
of chronic ICV AM251 results in an acute anhedonic state, lasting a maximum of 12 hours into administration.

Taste Reactivity

Unadjusted and baseline-adjusted taste reactivity data are presented in Figures 7 and 8 respectively. Neither ANOVA revealed any significant effects on either of the two test days. This may indicate that chronic AM251 did not influence taste reactivity; however, as the primary effect AM251 had on HFCS consumption was found on day 1 of drug administration, it is possible that the absence of any difference in TR on day 2 or 10 may be due to the timing of the tests.
EXPERIMENT 2

Introduction

Based on the results of the first experiment, Experiment 2 was designed with the aim of replicating and better characterizing the acute effect revealed in Experiment 1. In order to accomplish this, animals underwent the same surgical and experimental manipulations outlined in Experiment 1, with the exception that animals received only a single microinfusion of AM251 or vehicle, as opposed to chronic administration via osmotic minipump. HFCS, food, and water intake, as well as taste reactivity, were subsequently assessed. For a timeline of the procedures utilized in Experiment 2, see Figure 10.

Methods

Sixteen experimentally-naïve male Sprague-Dawley rats initially weighing 225-250 grams were used in this experiment (Charles River Laboratories, St Constant, Quebec). Animals were single-housed under the same conditions as outlined in Experiment 1. After a minimum of 5 days of habituation, animals were implanted with an intracranial cannula situated in the right lateral ventricle and an intraoral cannula for taste reactivity testing as previously described.

Following a 5-day recovery period, animals were allowed 24h in-cage ad libitum access to 50% HFCS solution for 8 days prior to experimental testing in order to ensure a stable baseline level of consumption and to eliminate novelty. Cannula placement and patency was confirmed via the angiotensin test conducted on day 5, and any animal which failed this test was automatically assigned to the sham group. A habituation trial for taste reactivity was conducted on day 6, and a baseline trial was conducted on day 7, as described in Experiment 1. Animals
with patent cannulas were then assigned to one of two groups, which were counterbalanced by body weight, baseline TR, and baseline HFCS intake over the last 3 days of measurement.

On day 9, animals in the first group received a 12.5 µg infusion of AM251 at a rate of 0.5 µl/min over a period of 1 minute, while animals in the second group received an equivalent dose of vehicle. After a 30-minute delay, animals underwent a TR test trial.

On day 10, treatment groups were reversed, except for the sham group. Animals were given a single infusion of 12.5 µg AM251 or vehicle at 0.5 µL/min over a period of 1 minute, then were immediately returned to their home cages. Pre-measured food, water, and HFCS were made available, and animals were allowed ad libitum access. Intake measurements were taken hourly for 5 hours post-infusion. Following all experimental manipulations, animals were humanely euthanized and blood and brain tissue was collected for later mRNA analysis (not presented).

Statistical Analysis

HFCS, water, and food intake were analyzed via two-way repeated-measures ANOVA. Taste reactivity data were converted into a percentage of baseline TR prior to being analyzed via one-way ANOVA. All statistical analyses were conducted using Sigmaplot version 12.2.

Results

Angiotensin Test

Six of the 16 animals used in this experiment did not fit the criteria as having passed the angiotensin test. As their cannula placement and patency could not be confirmed, these animals were assigned to the sham group.
Combining Controls

As was the case in Experiment 1, no significant differences were found between controls (vehicle and sham) with respect to any of the metrics assessed in Experiment 2. Consequently, the vehicle and sham groups were combined to form a single “control” group.

Unadjusted Intake

Unadjusted HFCS intake data up to 5 hours post-administration is presented in Figure 8. Results revealed a main effect of time \([F(4,56) = 7.650, \ p < 0.001]\). However, no effect of drug was found, nor was there an interaction.

Water and food intake are presented in Figures 9 and 10 respectively. The ANOVA for water intake revealed a main effect of time \([F(4,56) = 3.161, \ p = 0.021]\), but no effect of condition, nor an interaction. The ANOVA for food intake revealed no significant effects.

Taste Reactivity

Unadjusted and baseline-adjusted taste reactivity data are presented in Figures 12 and 13 respectively. No significant difference was found between treatment groups in either analysis. This may suggest that ICV administration of AM251 does not significantly influence hedonic processing.
GENERAL DISCUSSION

Summary

In Experiment 1, rats received 0, 1.25, 12.5, or 300 µg/day AM251 (approx. 0, 0.052, 0.52, and 12.5 µg/h respectively) suspended in 1:1 DMSO:PEG400 via subcutaneous osmotic minipump, or received sham implantation surgery. *Ad lib* HFCS, water, and food intake were measured 4 times daily for 10 days. TR was assessed on day 10 of habituation (baseline), as well as day 2 and day 10 of administration. The highest dose of ICV AM251 significantly reduced HFCS intake versus the medium-dose group and control group on day 1 of drug administration when compared to baseline. Conversely, no significant change was observed in either food or water intake in any drug group, nor was there any apparent effect on TR. This selective reduction in HFCS intake suggests that the highest dose of AM251 may have induced an acute anhedonic state.

Experiment 2 was conducted in order to examine further the possibility of an acute anhedonic state induced by ICV AM251. In the second experiment, novel animals were given an acute microinfusion of 12.5 µg AM251, vehicle, or a sham infusion and, after a 30 minute delay, TR to intraoral HFCS was assessed. The next day, drug and vehicle groups were reversed, such that half the patent animals were given a single 12.5 µg infusion of AM251 in DMSO:PEG400, while the other half received a vehicle infusion. HFCS, food, and water intake were assessed hourly for 5 hours post-infusion. When administered acutely, the same dose of AM251 capable of suppressing HFCS intake in Experiment 1 did not suppress HFCS intake in Experiment 2. Further, food and water intake, as well as TR, were likewise unaffected.
The results of these two experiments combined suggest that chronic, central administration of CB₁ inverse agonist AM251 at 12.5 µg/hour to the lateral ventricles can elicit an acute anhedonic state in rats, as evidenced by a selective reduction in palatable food intake. However, an acute infusion of the same dose is insufficient to induce the same effect. These results further implicate the central CB₁ receptor in reward processing and in clinical anhedonia.

Central CB₁ Receptor & Reward Processing

The primary aim of the present study was to establish whether chronic ICV AM251 is capable of inducing a persistent anhedonic state independent of any peripheral mechanisms, particularly nausea. Experiment 1 does confirm this to some degree: chronic administration of AM251 to the lateral ventricles does appear to have selectively reduced ad lib intake of a highly palatable food (HFCS). The selective nature of this suppression suggests that central CB₁ inverse agonism does induce anhedonia and, consequently, adds to the growing body of evidence which indicates the central endocannabinoid system plays a key role in reward processing and the development of anhedonia.

However, the effect observed in Experiment 1 was unexpectedly short-lived, lasting up to a maximum of 12 hours into chronic administration. Systemic studies of inverse agonists have found drug effects lasting up to 21 days of chronic administration via daily i.p. injection, as well as when administered continuously via subcutaneous osmotic minipump (Gessa et al., 2006; Rahn et al., 2014). One potential explanation may be that continuous central administration of AM251 to the lateral ventricles leads to the rapid development of tolerance. Tolerance to cannabinoids has been observed systemically and centrally, and chronic administration of rimonabant has been associated with a widespread down-regulation in CB₁ receptors (Martín-
García et al., 2010). A number of studies have indicated that tolerance to the feeding effects of agonists and inverse agonists typically develops 3-7 days into chronic systemic administration (Colombo et al., 1998; Maccioni, Pes, Carai, Gessa, & Colombo, 2008; Rigamonti, Giordani, Bonomo, Cella, & Müller, 2006). However, chronic studies typically administer the drug only once per day, rather than continuously, as is the case with an osmotic minipump. It may be the case, therefore, that the frequency of administration led to a more rapid development of tolerance than would otherwise be expected.

The results of Experiment 1 are generally consistent with a number of studies which have found that systemic administration of CB₁ inverse agonists negatively impacts feeding (Beyer et al., 2010; Higgs et al., 2003; Miner et al., 2008). However, there is some contention within the literature as to whether this systemic suppression of intake is specific to highly-palatable food, or whether CB₁ inverse agonism suppresses intake of all types of diets. While there is evidence that systemic CB₁ inverse agonism selectively reduces responding for highly palatable food (Droste et al., 2010), other studies have found that systemic administration also suppresses intake and responding for regular chow (McLaughlin et al., 2003; Miner et al., 2008). In contrast, central administration of AM251 did not appear to influence regular chow or water intake within the present study.

These disparate results in feeding and food-motivated behaviour in response to CB₁ inverse agonism may be the result of two distinct sites of action: one peripheral, and one central. Indeed, a number of other findings likewise indicate that this may be the case. For example, Gessa et al. (2006) found that chronic systemic rimonabant yielded a reduction in both regular chow and palatable food intake; however, while regular chow intake returned to normal 3-5 days
into treatment, palatable food intake remained suppressed for up to 21 days into administration. This suggests that the two forms of feeding suppression may be distinct in their mechanisms. Similarly, while chronic systemic administration of AM251 has been shown to suppress operant responding for regular chow, chronic ICV administration does not appear to have the same effect (McLaughlin et al., 2003; Sink et al., 2009). Further, deafferentation of the vagal nerve abolishes the suppression of regular chow intake elicited by systemic rimonabant, suggesting this intake suppression may be the result of the drug’s action on peripheral receptors within the gut (Gomez et al., 2002). While putative peripherally-restricted CB₁ inverse agonists have recently been synthesized (Hung et al., 2010), they have not yet been examined in vivo with respect to feeding behaviour. However, peripherally-restricted CB₁ antagonists, which block the receptor but do not induce their own effects, have been studied within this context, and have been shown to suppress feeding behaviour for several different diets, including regular chow (Loverme et al., 2008; Randall et al., 2010). These results, coupled with the present study’s evidence that central AM251 selectively suppresses highly palatable food intake, suggest that the endocannabinoid system’s influence on feeding behaviour may be the result of two different mechanisms of action, one involving central CB₁ receptors, resulting in palatable food suppression as a result of anhedonia, and one involving peripheral receptors, resulting in broader feeding suppression.

A likely site of action for the central effect of AM251 observed in the present study is within the mesolimbic DA system. The CB₁ receptor’s inhibitory action on glutamatergic and GABAergic neurons appears to play a modulatory role on DA activity within the mesolimbic pathway (Martín et al., 2008; M. Melis et al., 2004; T. Melis et al., 2007). Systemic CB₁ inverse agonists are associated with decreased DA activity in response to pleasurable stimuli, including highly palatable food (T. Melis et al., 2007). The CB₁ receptor also appears to interact with the
\(\mu\)-opioid system within the mesolimbic pathway, which is likewise implicated in hedonic reward processing with respect to both food and drugs of abuse (Kelley et al., 2002). Administration of a CB\(_1\) inverse agonist directly to the NAc can negate the increased DA activity elicited by a \(\mu\)-opioid agonist in a manner similar to a \(\mu\)-opioid antagonist (Skelly, Guy, Howlett, & Pratt, 2010). Further, co-administration of a CB\(_1\) agonist and a \(\mu\)-opioid agonist results in a greater increase on palatable food intake than when either is administered alone (Skelly et al., 2010). It seems, therefore, that while the present study’s site of administration was relatively nonspecific, the suppression of palatable food intake observed in Experiment 1 is likely due to impairment in reward processing resulting from AM251’s suppression of DA activity within mesolimbic structures.

The results of Experiment 1 indicate that central administration of AM251 is indeed able to induce anhedonia, as evidenced by the selective reduction of HFCS intake. This may be due to AM251 suppressing DA activity in response to palatable food consumption within the mesolimbic DA pathway. Further, the present study contributes to the growing body of evidence that CB\(_1\) inverse agonist-mediated feeding suppression is mediated by the drug’s influence on both peripheral and central mechanisms. Finally, the short-lived nature of AM251’s suppression of HFCS intake may be indicative of a rapid tolerance due to the frequency of administration used in the present study.

Experiment 2, however, did not replicate the results of Experiment 1: acute ICV AM251 did not significantly impact HFCS intake, as was expected. This may be due to the dosage used: only the highest dose of AM251 significantly reduced HFCS intake within the chronic study. Further, chronic administration via osmotic minipump results in a steady, continuous flow of
drug into the lateral ventricles, as opposed to a discrete microinfusion. It is possible, therefore, that this continuous administration allowed for AM251 to accumulate within the brain until the dose was sufficient to suppress HFCS intake. Alternatively, it may be the case that a discrete infusion is insufficient to elicit anhedonia; rather, it is the persistent action of the drug on central CB$_1$ receptors which results in the reduced sensitivity to reward observed in this study.

Finally, taste reactivity to HFCS was not found to have been influenced by either acute or chronic AM251. This result was ultimately unexpected; Jarrett, Scantlebury, & Parker (2007) found systemic AM251 reduced taste reactivity to a 32% sucrose solution. Further, Limebeer et al. (2010) found that the same dose of ICV AM251 utilized in this study significantly reduced tongue protrusions in response to intraoral infusion of saccharin. However, there may be a few reasons why this effect was not replicated in the present study. Firstly, in Experiment 1, taste reactivity was tested on day 2 and day 10 of drug administration, while the effect observed on HFCS intake was limited to the first 12 hours of day 1. As a result, it is possible that tolerance had already developed to the drug by the time the first TR test trial was conducted. Further, while the present study did not replicate the results of Limebeer et al. (2010), animals in the present study had in-cage access to HFCS right up until TR testing. Neath and colleagues (2010) found that hedonic response to intraoral infusion of a 0.5% saccharin solution decreased over the course of exposure, suggesting satiation. It may be the case, therefore, that prior exposure to HFCS in-cage influenced the animals’ TR response to intraoral HFCS infusion. Finally, while the formulation of HFCS used in the present study shares a similar glucose/fructose ratio to sucrose, the former can contain up to 3% polycose, which rats are capable of detecting, and which may have influenced its palatability and/or reward factor. (Sclafani & Mann, 1987).
Limitations & Future Considerations

There are a few limitations to this study. First and foremost, the number of animals in each experimental group is quite variable. This is due in part to the high number of animals (17 of 42) with intracranial cannulas that could not be verified as correctly placed and patent, which were consequently assigned to the sham group. This could potentially be rectified in the future by implanting the intracranial cannula, catheter tubing, and minipump simultaneously. The continuous infusion of drug solution through the intracranial cannula may help to prevent blockages from forming. However, there are a few potential disadvantages with this solution: firstly, patency and placement cannot be confirmed prior to drug administration. Secondly, any potential side effects of intracranial cannula implantation cannot be easily dissociated from drug effects, as administration begins immediately after implantation. Another potential alternative would be to use straight intracranial cannulas versus the 90-degree cannulas used in the present study; however, this would likely increase the risk of the catheter detaching from the cannula.

Likewise, it is difficult to confirm whether the drug adequately entered the brain via the minipump. Numerous measures were taken to ensure the drug was delivered properly: cannula patency was assessed via an angiotensin test, minipumps were opened and examined after removal to ensure their contents had been expressed, and the connection points between minipump, tubing, and intracranial cannula were secured during surgery and monitored for detachment throughout treatment. However, it is still possible that some animals experienced leakage, and subsequently did not receive the full dose of the drug.

Further, intake measurement during this study could have been more precise: while measurement times were drawn from observations of circadian rhythm, measurements were
taken manually, and intervals varied greatly by time of day. This interference could potentially have influenced the animals’ natural feeding behaviour, and the varied intervals between measurements make it difficult to draw any further conclusions regarding the effect observed within the first 12 hours of AM251 administration. Future studies would benefit from the use of cages equipped with automated food and fluid measurement, such that intake can be assessed precisely at regular intervals with minimal disturbance of the animals.

Finally, the number of habituation days between Experiment 1 and Experiment 2 differed: in Experiment 1, animals had in-cage access to HFCS for 13 days prior to drug administration, while in Experiment 2, animals were allowed in-cage access for 8 days prior to administration. This difference in exposure may have influenced subsequent HFCS intake during the drug administration phase. However, all animals acquired *ad-lib* HFCS consumption by the end of the habituation period in both experiments, and mean intake did not differ between groups or across experiments by the final day of habituation, suggesting that intake was equivalent between experiments. Nevertheless, future studies may benefit from replicating Experiment 2 with a full 13 habituation days in order to eliminate this potential confound.

Based on the results of the present study, there are a few other potential directions for future research to examine. For instance, it would be beneficial to replicate Experiment 2 with a higher dose of AM251: Limbeer et al. (2010) used up to 125 µg AM251, while the present study used a maximum of one-tenth the dose. Further, it would be beneficial to replicate both experiments using a more commonly-studied sugar, such as sucrose, in order to determine whether the palatability and, consequently, the TR response to HFCS is significantly impacted by its composition. Another potential option would be to administer AM251 to the lateral
ventricles chronically via a single intracranial infusion each day as in Sink et al. (2009), rather than via minipump, and to assess its effect on palatable food intake. This may help to prolong the neurological effects before the onset of tolerance. Further, administration of AM251 directly to reward-related regions, such as the VTA and/or NAc, may aid in pinpointing the brain regions involved in the acute anhedonic state observed in Experiment 1. Finally, future experiments should examine the effect of ICV AM251 on taste reactivity to HFCS independent of any prior in-cage exposure.

**Conclusions**

Overall, the highest dose of chronic ICV AM251 produced a significant, selective decrease in HFCS intake within the first 12 hours of administration. However, when replicated acutely, the same dose did not precipitate a similar decrease in HFCS intake. Further, taste reactivity remained unaffected by either chronic or acute AM251 administration. Taken together, these results suggest that 12.5 µg AM251, when administered chronically to the lateral ventricles, may induce an acute anhedonic state detectable via changes in palatable food consumption. This effect is likely the result of AM251’s action within the mesolimbic DA system, and further implicates the central CB₁ receptor in hedonic processing and in clinical anhedonia.
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FIGURE LEGENDS

Figure 1

A timeline of the surgical and experimental procedures carried out in Experiment 1.

Figure 2

Mean (SEM) percent of baseline HFCS intake on select days of chronic ICV administration.

* p < .05.

Figure 3

Mean (SEM) percent baseline water intake on select days of chronic ICV administration.

Figure 4

Mean (SEM) percent of baseline food intake on select days of chronic ICV administration.

Figure 5

Mean (SEM) hourly HFCS intake at key intervals throughout day 1 of chronic ICV administration. * p < .05.

Figure 6

Mean (SEM) hourly water intake at key intervals throughout day 1 of chronic ICV administration.

Figure 7

Mean (SEM) hourly food intake at key intervals throughout day 1 of chronic ICV administration.
Figure 8

Mean (SEM) number of tongue protrusions (hedonic response) as measured via taste reactivity on day 2 and day 10 of chronic ICV administration.

Figure 9

Mean (SEM) percent of baseline tongue protrusions (hedonic response) as measured via taste reactivity on day 2 and day 10 of chronic ICV administration.

Figure 10

A timeline of the surgical and experimental procedures carried out in Experiment 2.

Figure 11

Mean (SEM) HFCS intake over 5 hours post-administration of either 12.5 µg AM251 or a control condition (vehicle + sham).

Figure 12

Mean (SEM) water intake over 5 hours post-administration of either 12.5 µg AM251 or a control condition (vehicle + sham).

Figure 13

Mean (SEM) food intake over 5 hours post-administration of either 12.5 µg AM251 or a control condition (vehicle + sham).

Figure 14
Mean (SEM) number of tongue protrusions (hedonic response) as measured via taste reactivity.

**Figure 15**

Mean (SEM) percent of baseline tongue protrusions (hedonic response) as measured via taste reactivity.
FIGURES

Figure 1
Figure 2

[Bar chart showing data for different days (Day 1, Day 2, Day 5, Day 10) with varying conditions (Control, 1.25 mg/kg BW, 2.5 mg/kg BW, 5 mg/kg BW).]
Figure 3
Figure 4
Figure 6

Chart showing water intake per hour (ml) with mean (SEM) across different conditions.
Figure 7
Figure 8
Figure 11
Figure 12
Figure 13

![Graph showing food intake over hours with two conditions: Control and 12.5 μg AM251. The y-axis represents mean (SEM) food intake in grams, and the x-axis represents hours. The graph shows a decrease in food intake from hour 1 to hour 2, followed by an increase in hour 3 for both conditions. From hour 3 to hour 5, the food intake for both conditions remains relatively constant. The error bars indicate the standard error of the mean.](image-url)
Figure 14

![Graph showing mean (SEM) number of protrusions comparing Control and 12.5 μg AM251 groups.](image-url)
Figure 15