

RESEARCH PAPER

The anti-nausea effects of CB₁ agonists are mediated by an action at the visceral insular cortex

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BACKGROUND AND PURPOSE

Conditioned gaping reactions reflect nausea-induced behaviour in rats. Cannabinoid 1 receptor (CB₁) agonists interfere with the establishment of nausea-induced conditioned gaping; however, it is not known if their effects are mediated by an action at peripheral or central CB₁ receptors.

EXPERIMENTAL APPROACH

We utilized the conditioned gaping model of nausea to evaluate the effect of peripheral and central administration of the peripherally restricted CB₁ agonist, CB13, on the establishment of LiCl-induced gaping in rats. We further evaluated the ability of HU-210 administered to the gustatory insular cortex (GIC) or visceral insular cortex (VIC) to interfere with LiCl-induced conditioned gaping and determined if this effect was mediated by CB₁ receptors.

KEY RESULTS

Central, but not peripheral, CB13 suppressed LiCl-induced conditioned gaping. Central administration of the potent CB₁ agonist, HU-210, delivered to the VIC, but not the GIC, suppressed the establishment of LiCl-induced gaping reactions, but not LiCl-induced suppression of hedonic reactions or conditioned taste avoidance. This pattern of results suggests that HU-210 delivered to the VIC prevented LiCl-induced nausea, but not learning *per se*. The suppression of LiCl-induced conditioned gaping by HU-210 was mediated by CB₁ receptors because it was prevented by co-administration of CB₁ antagonist/inverse agonist, AM-251, into the VIC. A high dose of AM-251 (20 µg) administered alone into the VIC did not produce conditioned gaping reactions.

CONCLUSIONS AND IMPLICATIONS

The nausea-relieving effects of CB₁ agonists, but not the nausea-inducing effects of CB₁ inverse agonists, are mediated, at least in part, by their action at the VIC in rats.

Abbreviations

2-HPβCD, 2-hydroxypropyl-β-cyclodextrin; CB₁, cannabinoid 1 receptor; CB13, naphthalene-1-yl-(4-pentyloxynaphthalen-1-yl)methanone; CTA, conditioned taste avoidance; GIC, gustatory insular cortex; HU-210, 3-(1,1'-dimethylheptyl)-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; THC, Δ⁹-tetrahydrocannabinol; TR, taste reactivity; VIC, visceral insular cortex

Introduction

The neurobiology of nausea is not well understood. One limitation to the study of nausea has been the lack of a reliable animal model. Although rats are incapable of vomit-

ing, there is considerable evidence that conditioned disgust reactions (most reliably gaping: rapid, large amplitude opening of the mandible with retraction of the corners of the mouth) elicited by an illness-paired flavour (e.g. Grill and Norgren, 1978a; Parker *et al.*, 2008) represent such a model.

Conditioned gaping reactions are measured in the taste reactivity (TR) test (Grill and Norgren, 1978a), during which an illness-paired flavour is intraorally infused across their tongues. Unlike the traditionally employed consumption test measure of conditioned taste avoidance (CTA), conditioned gaping is exclusively produced by emetic treatments, but not by rewarding drugs (Parker, 1995; Parker *et al.*, 2008). Furthermore, anti-emetic treatments selectively interfere with the establishment of conditioned gaping, but not CTA (e.g. Grant, 1987; Limebeer and Parker, 2000; Tuerke *et al.*, 2011). Therefore, conditioned gaping in rats, unlike CTA, represents nausea-induced behaviour (Parker *et al.*, 2008). At low doses, cannabinoid 1 receptor (CB₁) agonists and drugs that enhance the activity of the endogenous cannabinoid system reduce not only vomiting (Darmani, 2001; Van Sickle *et al.*, 2001; Parker *et al.*, 2004) but also conditioned disgust in rats in a manner that suggests they reduce nausea-like behaviours (Parker and Mechoulam, 2003; Parker *et al.*, 2003; Cross-Mellor *et al.*, 2007; Sticht *et al.*, 2011).

As there is evidence that both central and peripheral CB₁ receptors may be involved in the control of emetic reactions (Van Sickle *et al.*, 2001; Darmani and Johnson, 2004), it is unclear whether the regulation of nausea by agonists of these receptors is peripherally or centrally mediated. Recently, a relatively peripherally restricted CB₁/CB₂ agonist, CB13, has been developed (Dziadulewicz *et al.*, 2007). The experiments reported here evaluated the effect of peripheral and i.c.v. administration of CB13 to prevent conditioned gaping in rats. As only i.c.v. administration of CB13 reduced LiCl-induced conditioned gaping, the CB₁ agonist HU-210 was microinfused into the visceral insular cortex (VIC) and gustatory insular cortex (GIC), and the effect on LiCl-induced conditioned gaping was evaluated. The insular cortex is a forebrain region that has been shown to be involved in the generation of human disgust (e.g. Calder *et al.*, 2001; 2007), conditioned disgust in rats (Kiefer and Orr, 1992) and nausea in humans and other animals (Kaada, 1951; Penfield and Faulk, 1955; Fiol *et al.*, 1988; Contreras *et al.*, 2007; Catenoix *et al.*, 2008). Finally, the potential of intracranial AM-251 delivered to the VIC to interfere with HU-210-induced attenuation of conditioned gaping was evaluated.

Methods

Animals

A total of 171 male Sprague-Dawley rats (Charles River Lab, St Constant, QC, Canada) weighing 300–380 g on the day of conditioning (11 rats were removed from final data analysis due to inaccurate cannula placement). The rats were individually housed in Plexiglas cages (48 × 26 × 20 cm) in the colony room at an ambient temperature of 21°C with a 12/12 reverse light/dark schedule (lights off at 07:00 h). All experimental manipulations were conducted during the dark phase of the cycle. Rats were provided with two clean paper towels (replenished during weekly cage changes) and a soft plastic container that was 14 cm long and 12 cm in diameter that remained in the home cage. They were maintained on food [Highland Rat Chow (8640)] and water *ad libitum* throughout the experiments. All procedures were approved by the Animal

Care Committee of the University of Guelph and adhered to the guidelines of the Canadian Council of Animal Care. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Drugs

LiCl was prepared in a 0.15 M solution with sterile water and was administered i.p. at a volume of 20 mL·kg⁻¹ (127.2 mg·kg⁻¹). Peripherally administered CB13 and Δ⁹-tetrahydrocannabinol (THC) were prepared in a vehicle of 45% 2-hydroxypropyl-β-cyclodextrin (2-HPβCD) with sterile water. Various groups were injected i.p. with CB13 at doses of 0.3 (prepared as 0.3 mg·mL⁻¹), 1 (prepared as 1.0 mg·mL⁻¹) and 3 mg·kg⁻¹ (prepared as 1.5 mg·mL⁻¹). At doses of 3 mg·kg⁻¹ or less, CB13 has been shown to have anti-hyperalgesic efficacy in a rat model of neuropathic mechanical hyperalgesia that is blocked by *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (SR141716A) with no indication of CNS effects as measured by catalepsy (a typical centrally mediated CB₁ agonist response). Furthermore, a 3 mg·kg⁻¹ dose had a C_{max} of 1.13 μM at 1 h post-delivery and a maximal brain concentration of 0.24 μmol·kg⁻¹ at 4 h post-dose (Dziadulewicz *et al.*, 2007). THC (3.0 mg·kg⁻¹, i.p.) was used as a positive control for the peripheral restriction of CB13 due to the ability of THC to cross the blood–brain barrier at this dose. Centrally (i.c.v.) administered CB13 was prepared in 100% dimethyl sulfoxide (DMSO) vehicle at concentrations of 0.3, 1.0 and 3.0 μg·μL⁻¹ and delivered at a rate of 1 μL·min⁻¹. For Experiment 2, HU-210 for intracranial (VIC and GIC) administration was prepared in a vehicle of 45% HPβCD at concentrations of 0.001 and 0.01 μg·μL⁻¹ and was delivered at a rate of 1 μL·min⁻¹. For Experiment 3, HU-210 was prepared in 45% HPβCD at a concentration of 0.02 μg·μL⁻¹ and AM-251 was prepared in 100% DMSO vehicle at a concentration of 2 μg·μL⁻¹. Both drugs were delivered at a rate of 0.5 μL·min⁻¹, with AM-251 being delivered 5 min before HU-210. For Experiment 4, AM-251 was prepared in 100% DMSO vehicle at concentrations of 0.1, 1.0, 10.0 and 20.0 μg·μL⁻¹ and was delivered at a rate of 1 μL·min⁻¹. All microinfusions were of 1 min duration with the injector left in place for 1 min post-infusion, before removal from the guide cannula. The obturator was replaced after each microinfusion. For Experiments 2a, 2b, 3 and 4, the microinfusions were delivered bilaterally. Our drug/molecular target nomenclature conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Surgery

In all experiments, the rats were surgically implanted with an intraoral cannula, as described in detail by Limebeer *et al.* (2010). In Experiments 1b–4, the rats were anaesthetized with isoflurane gas and prepared for intracranial surgery by shaving a strip of skin between the ears (2.5–3 cm long), cleaning the skin and injecting Carprofen (5 mg·kg⁻¹, i.p.) and a topical anaesthetic (0.1 mL, s.c.) on either side of the skull (Marcaine; Hospira, Montreal, QC, Canada). Verification of a surgical plane of anaesthesia in the rat was done by demonstration of a lack of a withdrawal reflex response in the hindlimb, decreased muscle tone and a slow, regular respira-

tory response. They were then stabilized in the flat skull position (according to Paxinos and Watson, 1998) in the stereotaxic frame and the skull was exposed. In Experiment 1b, the rats had a unilateral indwelling guide cannula surgically implanted in the lateral ventricle. The stainless steel guide cannula (22 G, 6 mm below pedestal) was lowered into either the left or the right lateral ventricle (counterbalanced among groups) using the following coordinates from Bregma: -1.0 mm anteroposterior (AP), ± 1.4 mm lateromedial (LM), -3.6 mm dorsoventral (DV) (Erb *et al.*, 2003). In Experiments 2a-4 (VIC) and 2b (GIC), the rats had bilateral indwelling guide cannulae implanted into the VIC (arms set at divergent 10° angle, relative to Bregma: AP -0.5 ; LM ± 5.0 ; DV -4.5 from the skull) or the GIC (relative to Bregma: AP $+1.5$, LM ± 5.0 , DV -4.5 from the skull). The guide cannulae were stabilized by six screws secured in the skull and dental cement. Once the dental cement had hardened, a stainless steel obturator was inserted into the guide cannulae to maintain patency. Following removal from the stereotaxic frame, the rats were surgically implanted with an intraoral cannula. They were then placed in a heated recovery area and monitored until they were ambulatory, at which time they were returned to the colony room. The rats were allowed to recover for 5 days following surgery and were monitored daily as previously described (Limebeer *et al.*, 2010).

Histology

Verification of cannula placement was determined by histological evaluation of tissue. The rats were deeply anaesthetized using an $85 \text{ mg}\cdot\text{kg}^{-1}$ injection of Euthansol (Intervet Canada Corp., Kirkland, QC, Canada), followed by transcardial perfusion with PBS (0.1 M) and 4% formalin. The brains were removed and stored at 4°C in 4% formalin solution for 24–48 h, after which they were placed in a 20% sucrose solution overnight at room temperature. The brains were then sliced in $60 \mu\text{m}$ sections using a CM1850 Leica cryostat and relevant sections were mounted on gelatin-subbed glass microscope slides. The tissue was stained with thionin 24 h later and examined for accurate cannula placement using a Leica MZ6 Stereomicroscope with a Leica DFC420 Digital Camera and Leica Application Suite software (Leica Microsystems Inc., Concord, ON, Canada). Any rat with improper cannula placement was excluded from the behavioural analysis.

Apparatus

Conditioning and testing was conducted in a square Plexiglas TR chamber ($26.5 \times 26.5 \times 12 \text{ cm}$) placed on a glass-topped table, with a mirror set at 45° below the glass top to facilitate videotaping the ventral surface of the rat while in the chamber. The rat's intraoral cannula was attached to the infusion pump (KDS100; KD Scientific Inc., Holliston, MA, USA) using a length of PE160 tubing fitted over the intraoral cannula that ran through a hole at the top of the chamber and connected to the pump. Orofacial responses were captured directly by the computer using the Roxio Videowave Premiere Suite 8 video capture program (Corel Corporation, Ottawa, ON, Canada) and a Sony DCR-HC28 Handycam camera (Sony Corporation, Toronto, ON, Canada).

For central administration of solutions, microinfusions were conducted using a 28 G injector that extended 2 mm beyond the guide cannula tip and was attached to a micro-

infusion pump (KDS101; KD Scientific Inc.) using Tygon tubing with a 0.1905 mm inner diameter (Cole-Parmer, Vernon Hills, IL, USA).

Procedure

Experiment 1: potential of systemic CB13 (Experiment 1a) or i.c.v. CB13 (Experiment 1b) to attenuate LiCl-induced conditioned gaping. Five days after recovering from surgery, the rats received an adaptation trial, during which they were individually placed in the TR chamber with their cannula attached to the infusion pump for fluid delivery. After 3 min in the TR chamber, water was infused into their intraoral cannula over a period of 3 min at a rate of $1 \text{ mL}\cdot\text{min}^{-1}$. Twenty-four hours later, the rats received a conditioning trial. In Experiment 1a, they were randomly assigned to pretreatment groups on the basis of dose of CB13 (with all *ns* in each experiment reported only for correct cannula placements): group vehicle (VEH; $n = 6$), group $0.3 \text{ mg}\cdot\text{kg}^{-1}$ CB13 ($n = 7$), group $1 \text{ mg}\cdot\text{kg}^{-1}$ CB13 ($n = 8$), group $3 \text{ mg}\cdot\text{kg}^{-1}$ CB13 ($n = 8$) and group $3.0 \text{ mg}\cdot\text{kg}^{-1}$ THC ($n = 7$). Thirty minutes before the conditioning trial, the rats were injected i.p. with the appropriate pretreatment drug. In Experiment 1b, the rats were randomly assigned to groups on the basis of pretreatment drug: VEH ($n = 7$), $10 \mu\text{g}$ CB13 ($n = 7$) or $30 \mu\text{g}$ CB13 ($n = 8$). Immediately before the conditioning trial, $1 \mu\text{L}$ of the pretreatment drug was microinfused and the injector was left in place for 1 min following the microinfusion. In both Experiments 1a and 1b, the rats were then placed in the TR chamber and 3 min later were intraorally infused with 0.1% saccharin for 3 min at a rate of $1 \text{ mL}\cdot\text{min}^{-1}$. Immediately following the saccharin infusion, the rats were injected with $20 \text{ mL}\cdot\text{kg}^{-1}$ of LiCl and returned to their home cage. Seventy-two hours later, the rats received the drug-free TR test trial, during which they were placed in the TR chamber for 3 min before receiving an intraoral infusion of 0.1% saccharin solution over a period of 3 min ($1 \text{ mL}\cdot\text{min}^{-1}$), while their orofacial reactions were recorded from the mirror beneath the chamber. On the following day, after being deprived of water for 16 h, the rats received a test of CTA. The test was a single-bottle test because the single-bottle test has been shown to be more sensitive to between-group differences than two-bottle choice tests, with water as an alternative (Batsell and Best, 1993). The two-bottle test is highly sensitive to the detection of even very weak CTA, producing floor effects that may mask between-group differences. The rats were given a single bottle of saccharin to consume for 30 min and the amount consumed was measured.

Experiment 2: potential of intracranial administration of HU-210 into the VIC (Experiment 2a) or the GIC (Experiment 2b) to attenuate LiCl-induced conditioned gaping. Experiments 2a and 2b were conducted similar to Experiment 1, except as indicated. Rats received adaptation training to the TR procedure exactly as in Experiment 1 on the sixth day following surgery. Unlike Experiments 1a and 1b, the groups received two conditioning trials (separated by 72 h) in Experiments 2a and 2b before TR testing. In Experiment 2a (VIC infusions), the rats were randomly assigned to the various groups: VEH ($n = 6$), $0.001 \mu\text{g}$ HU-210 ($n = 6$) and $0.01 \mu\text{g}$ HU-210 ($n = 8$). In Experiment 2b (GIC), they were randomly assigned to the following groups: VEH ($n = 6$), $0.001 \mu\text{g}$ HU-210 ($n = 6$) and

0.01 µg HU-210 ($n = 7$). On each of the two conditioning trials, the rats were bilaterally microinfused with 1 µL of the pretreatment drug at a rate of 1 µL·min⁻¹, with the injector left in place for 1 min. Immediately following the microinfusion, the rats were placed in the TR chamber and after 3 min, they received a 3 min intraoral infusion of 0.1% saccharin solution at a rate of 1 mL·min⁻¹, with their orofacial reactions videotaped. They were all injected with LiCl immediately upon removal from the TR chamber. The drug-free TR test occurred 72 h following the second conditioning trial. The rats received a CTA test on the following day as in Experiments 1a and 1b.

Experiment 3: potential of AM-251 to reverse the suppression of conditioned gaping by HU-210, both delivered to the VIC. Experiment 3 aimed to determine if the suppression of LiCl-induced nausea by HU-210 delivery to the VIC was CB₁-mediated. The procedures of Experiment 3 were identical to those of Experiment 2a, except that the rats received two pretreatment microinfusions into the VIC immediately before each of the saccharin-LiCl pairings on the conditioning trials according to the following random group assignment: VEH-VEH ($n = 6$), AM2-51-VEH ($n = 7$), VEH-HU210 ($n = 7$) and AM-251-HU210 ($n = 7$). The microinfusions were delivered at a rate of 0.5 µL·min⁻¹ for 1 min and the injector was left in place for 1 min, making the total volume of liquid delivered to the VIC 1 µL.

Experiment 4: potential of AM-251 delivered to the VIC to produce conditioned gaping. CB₁ inverse agonists produce nausea in humans (Janero and Makriyannis, 2009) and conditioned gaping in rats (McLaughlin *et al.*, 2005) when explicitly paired with saccharin solution. Experiment 4 aimed to determine if the nausea produced by AM-251 is mediated by its action in the VIC. In Experiment 4, the rats were treated as in Experiment 2a, except they did not receive pretreatment drugs or LiCl. Instead, on each conditioning trial immediately following the intraoral infusion of saccharin solution, the rats received VIC microinfusions of VEH ($n = 8$), 0.1 µg AM-251 ($n = 5$), 1.0 µg AM-251 ($n = 8$), 10 µg AM-251 ($n = 8$) or 20 µg AM-251 ($n = 7$). Because the two-bottle preference test is more sensitive to the detection of a weak CTA than the one-bottle test (Batsell and Best, 1993), following 16 h of water deprivation, on the day following the TR test trial, the rats were given a graduated tube with saccharin and one with water for a total of 6 h to detect a CTA with measures taken at 30, 120 and 360 min.

Behavioural measures

The video recordings obtained were scored using the Observer (Noldus Information Technology, Sterling, VA, USA) event recording programme for the following behaviours: the number of *gaping reactions* (rapid, large amplitude opening of the mandible with retraction of the corners of the mouth) was counted during the 3 min infusions. The number of seconds that the rats displayed *hedonic reactions* of mouth movements (movement of the lower mandible without opening the mouth) and tongue protrusions (extensions of the tongue out of the mouth) was also measured and the total of the two measures was used as the hedonic score. The mean

amount of saccharin consumed during the 30 min CTA test in Experiments 1–3 served as a measure of CTA. The mean saccharin preference ratios [amount of saccharin consumed/(amount of saccharin + water consumed)] at each interval of testing served as a measure of CTA in Experiment 4.

Data analysis

In each experiment, the mean number of gapes and periods (measured in s) of hedonic reactions displayed by the rats on the conditioning trial(s) and on the test trial were entered into mixed-factor ANOVAs with the between-group factor of pretreatment condition and the within-group factor of trial. Bonferroni *post hoc* comparison tests were conducted for statistically significant effects. The amount of saccharin solution consumed during the 30 min CTA test in each of Experiments 1–3 were entered into a one-way ANOVA and the mean saccharin preference ratio in Experiment 4 was analysed as a 5 (dose) by 3 (time) mixed-factor ANOVA. Statistical significance was defined as $P < 0.05$.

Results

Experiment 1: effect of systemic (1a) or i.c.v. (1b) CB₁₃ on LiCl-induced conditioned gaping

Systemic administration of the peripherally restricted CB₁ and CB₂ agonist, CB₁₃, failed to attenuate the establishment of LiCl-induced conditioned gaping reactions, suppression of hedonic reactions or CTA. Systemic administration of THC attenuated LiCl-induced conditioned gaping reactions but did not interfere with suppression of hedonic reactions or CTA. Figure 1 presents the mean ($\pm$ SEM) number of gapes (upper graph) and mean duration (s; $\pm$ SEM) of hedonic reactions (lower graph) displayed by each pretreatment group during the conditioning trial and the drug-free TR test trial in Experiment 1. The 5×2 mixed-factor ANOVA of the gaping data revealed a significant trial effect, $F(1,31) = 141.0$; $P < 0.001$ and a significant trial by pretreatment interaction, $F(4,31) = 5.7$; $P = 0.001$. Simple main effect analysis of the interaction revealed that rats displayed gaping only on the test trial, $F(4,31) = 11.43$; $P < 0.001$. Subsequent Bonferroni *post hoc* comparisons revealed that rats pretreated with THC expressed significantly fewer conditioned gaping reactions than rats in any other pretreatment group, which did not differ from each other ($P < 0.025$). Analysis of the hedonic reaction score also revealed only an effect of trials, $F(1, 31) = 199.6$; $P < 0.001$, with rats showing fewer hedonic reactions during the test than during conditioning. The single-factor ANOVA of the mean amount consumed in the CTA test revealed no significant pretreatment group effect, $F(4,31) = 0.3$ (data not shown).

When administered i.c.v. to the lateral ventricle, a dose of 30 µg, but not 10 µg, of CB₁₃ interfered with the establishment of LiCl-induced conditioned gaping; however, no dose of CB₁₃ attenuated suppressed hedonic reactions or CTA. Figure 2 presents the mean ($\pm$ SEM) number of gapes (upper graph) and duration (s; $\pm$ SEM) of hedonic reactions (lower graph) displayed by the various groups in Experiment 1b during conditioning and testing. The 3×2 mixed factors

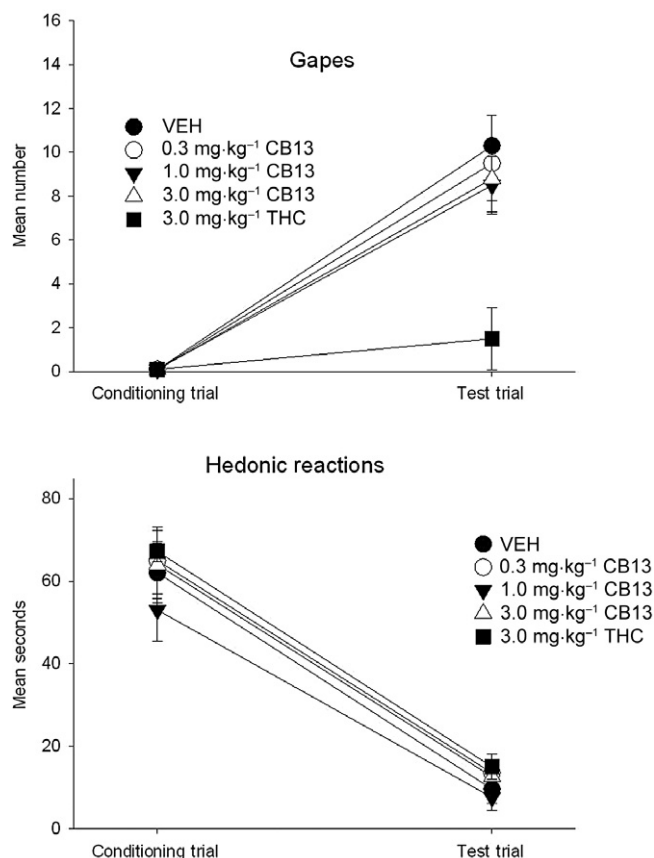


Figure 1

The mean ($\pm$ SEM) number of gaping responses (upper graph) and duration (s) of hedonic reactions (lower graph) expressed during the conditioning and test trials following systemic administration of CB13 or THC. The peripherally restricted CB₁/CB₂ agonist and THC did not alter hedonic reactions during the conditioning trial and did not modify suppressed hedonic reactions at test. THC, but not CB13, suppressed LiCl-induced conditioned gaping reactions at test.

ANOVA of the number of gapes revealed a significant pretreatment $\times$ trials interaction, $F(2, 19) = 4.5$; $P = 0.026$. Subsequent LSD comparison tests revealed that on the TR test trial rats pretreated with 30 μ g CB13 displayed fewer gapes during testing than either other group ($P < 0.05$). The 3×2 ANOVA for the hedonic reaction scores revealed only a significant effect of trials, $F(1, 19) = 161.4$; $P < 0.001$. Regardless of pretreatment conditions, rats showed fewer hedonic reactions during the TR test trial than during conditioning. The single factor ANOVA of the mean amount consumed in the CTA test revealed no significant effect of pretreatment group, $F(2, 19) = 0.2$ (data not depicted). Figure 3 presents a representative photomicrograph of the track of an i.c.v. cannula.

Experiment 2: effect of intracranial administration of HU-210 to the VIC (2a) and the GIC (2b) on LiCl-induced conditioned gaping

When administered to the VIC, but not to the GIC, HU-210 blocked the establishment of LiCl-induced conditioned

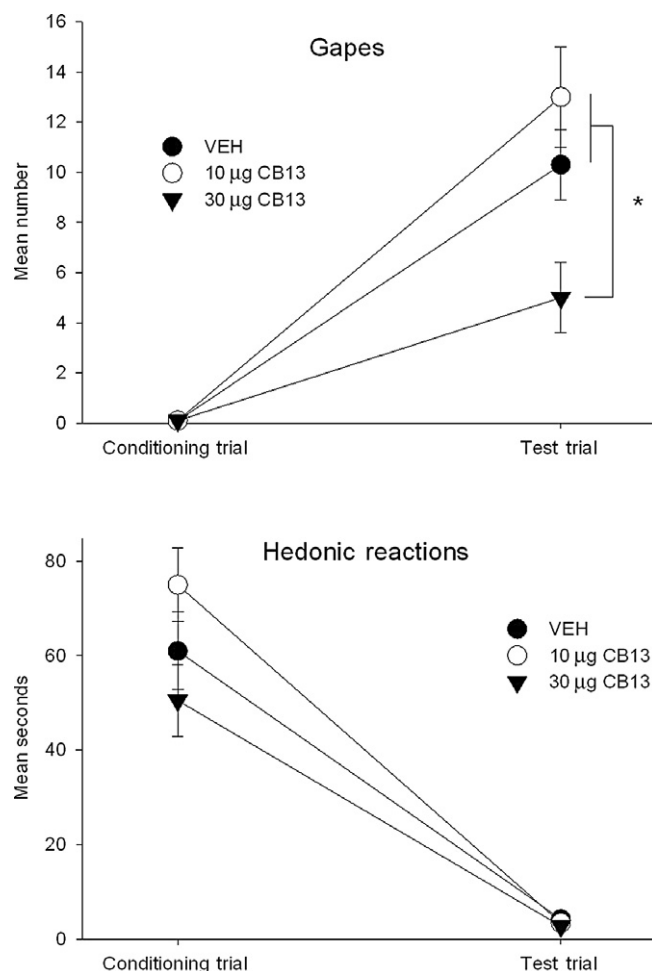


Figure 2

The mean ($\pm$ SEM) number of gaping responses (upper graph) and duration (s) of hedonic reactions (lower graph) expressed during the conditioning and test trials following central (i.c.v.) administration of CB13. The peripherally restricted CB₁/CB₂ agonist attenuated LiCl-induced conditioned gaping reactions in a dose-dependent manner; the 30 μ g group expressed significantly fewer gaping responses than the vehicle or 10 μ g groups (* $P < 0.05$). Centrally administered CB13 did not alter hedonic reactions during the conditioning trial and failed to attenuate suppressed hedonic responding during the test trial.

gaping at both 0.001 and 0.01 μ g. Figure 4 presents the mean ($\pm$ SEM) number of gapes displayed by the groups pretreated with VEH, 0.001 or 0.01 μ g of HU-210 in Experiment 2a (VIC) and Experiment 2b (GIC). The 3×3 mixed-factor ANOVA of gaping in Experiment 2a revealed significant effects of pretreatment, $F(1,17) = 4.0$; $P = 0.038$, trial, $F(2,34) = 27.0$; $P < 0.001$ and pretreatment $\times$ trial, $F(4,34) = 4.3$; $P = 0.006$. Subsequent Bonferroni *post hoc* comparison tests revealed that in the test trial only, the rats pretreated with VEH displayed significantly more gaping reactions than the rats pretreated with either 0.001 or 0.01 μ g HU-210 ($P < 0.05$). The ANOVA for the mean number of gapes displayed in Experiment 2b (GIC) revealed only a significant trial effect, $F(2,32) = 30.2$; $P < 0.001$; across all pretreatment groups, the rats displayed significantly more gaping across trials.

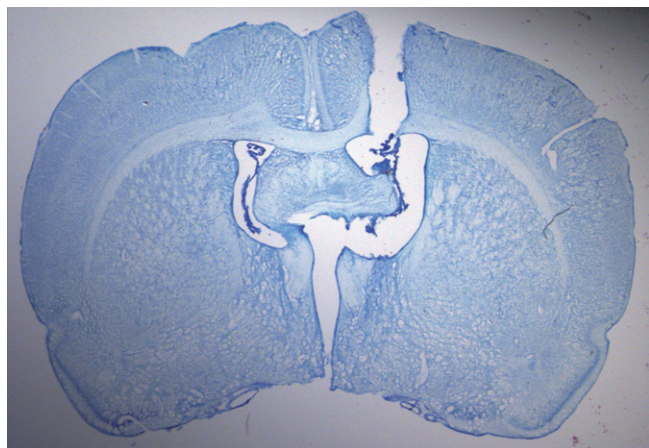


Figure 3

Representative photomicrograph of an i.c.v. cannula track in Experiment 1B.

Whether administered to the VIC or the GIC, HU210 did not reduce LiCl-induced suppression of hedonic reactions or CTA. Figure 5 presents the mean ($\pm$ SEM) number of seconds that the rats displayed hedonic reactions in Experiments 2a (VIC) and 2b (GIC). The 3×3 mixed-factor ANOVAs of hedonic reaction scores revealed only a significant effect of trials for Experiment 2a (VIC), $F(2,34) = 52.6$; $P < 0.001$, and for Experiment 2b (GIC), $F(2,32) = 116.9$; $P < 0.001$. Regardless of the pretreatment condition, the interaction was not significant. The single-factor ANOVAs of the mean amount of saccharin consumed in the CTA tests in Experiments 2a (VIC) and 2b (GIC) revealed that the pretreatment groups did not have a significantly different score for CTA [Experiment 2a: $F(2,17) = 1.9$; Experiment 2b: $F(2,16) = 0.5$] (data not depicted).

Experiment 3: effect of co-administration of AM-251 and HU-210 in the VIC on LiCl-induced conditioned gaping

Central administration of AM-251 to the VIC before HU-210 prevented the suppression of LiCl-induced conditioned gaping; however, as in Experiments 1 and 2, no pretreatment condition modified suppressed hedonic reactions or CTA. Figure 6 presents the mean ($\pm$ SEM) number of gapes (upper graph) and the mean ($\pm$ SEM) number of seconds that the rats displayed hedonic reactions (lower graph) in Experiment 3. The 4×3 mixed-factor ANOVA of gaping revealed significant effects of group, $F(3,23) = 3.6$; $P = 0.03$, trials, $F(2,46) = 39.3$; $P < 0.001$, and group $\times$ trials, $F(6, 46) = 2.4$; $P = 0.04$. Subsequent Bonferroni *post hoc* comparison tests revealed that on the test trial only, group VEH-HU210 displayed significantly fewer gapes than any other group ($P < 0.05$) with no other significant effects. The 4×3 mixed-factor ANOVA of hedonic reactions revealed only a significant effect of trials, $F(2,46) = 139.0$; $P < 0.001$. The single-factor ANOVA of the CTA test revealed no group differences in the strength of the CTA, $F(3,23) = 1.1$ (data not depicted).

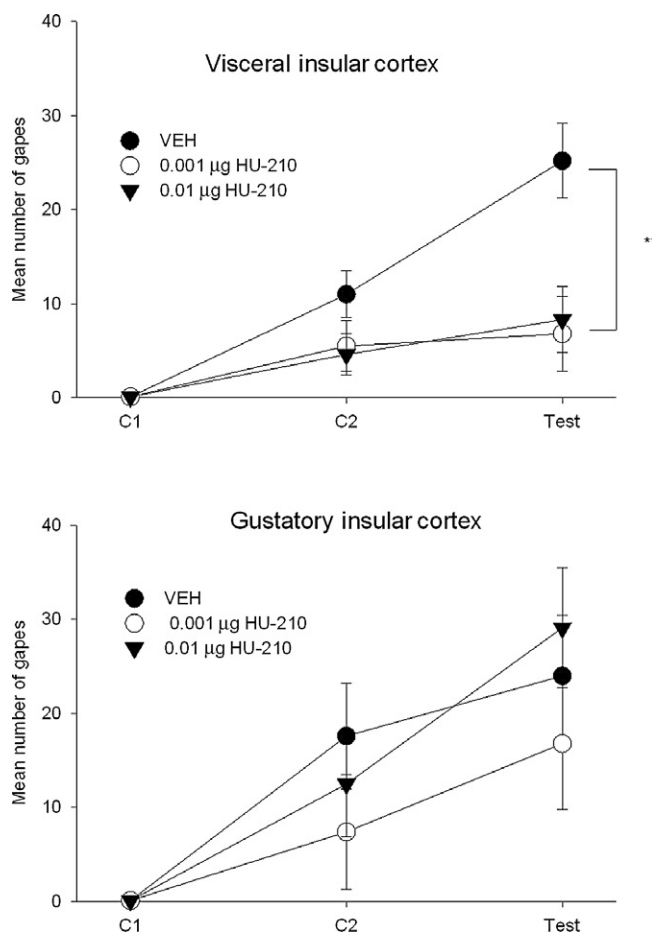


Figure 4

The mean ($\pm$ SEM) number of gaping responses expressed during the conditioning and test trials following microinfusion of HU-210 to the VIC (upper graph) or GIC (lower graph). This potent CB₁ agonist attenuated conditioned gaping responses at both doses (** $P < 0.01$) only when microinfused into the VIC. HU-210 did not interfere with LiCl-induced gaping responses at either dose when administered to the GIC.

Experiment 4: does VIC administration of AM-251 produce nausea?

When AM-251 was centrally administered to the VIC, it did not produce conditioned gaping reactions, suppress hedonic reactions or CTA at doses as high as 20 μ g. Figure 7 presents the mean ($\pm$ SEM) number of gapes (upper graph) and the mean ($\pm$ SEM) number of seconds that the rats displayed hedonic reactions (lower graph) in Experiment 4. The 5×3 mixed-factor ANOVA of gaping and hedonic reactions revealed only a significant effect of trial [gaping: $F(2,62) = 3.4$; $P = 0.04$; hedonic reactions: $F(2, 62) = 4.0$; $P = 0.023$] with gaping higher and hedonic reactions lower on the test trial than the other trials. The mean ($\pm$ SEM) preference ratios at 30, 120 and 360 min for each group are depicted in Figure 8. The 5×3 mixed-factor ANOVA revealed only a significant effect of time, $F(2,62) = 35.3$; $P < 0.001$; the rats had a higher saccharin preference ratio at the 360 min measure than at the earlier measure, but this did not differ among the groups.

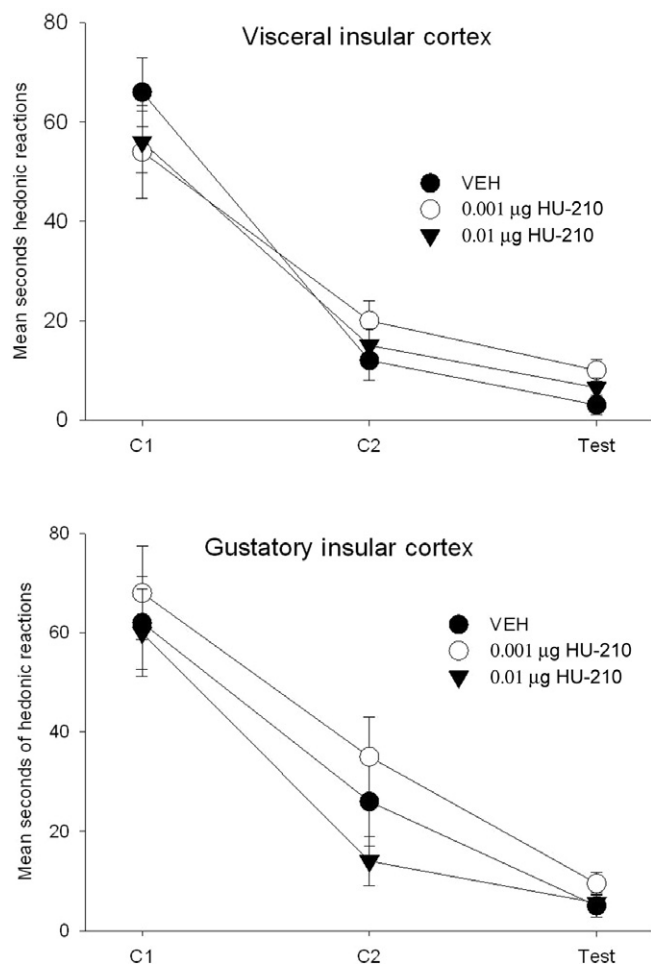


Figure 5

The mean ($\pm$ SEM) duration (s) of hedonic reactions expressed during the conditioning and test trials following microinfusion of HU-210 to the VIC (upper graph) and GIC (lower graph). HU-210 did not alter hedonic reactions during the first conditioning trial and failed to attenuate suppressed hedonic reactions during the second conditioning or test trials when administered to either the VIC or the GIC.

Discussion

There is considerable evidence indicating that CB₁ agonists are involved in the regulation of both nausea and vomiting in animal models (for complete review, see Parker *et al.*, 2011). Here, we provide evidence that the potent CB₁ agonist, HU-210, acts at the visceral region of the insular cortex to relieve the nausea-inducing effect of systemically administered LiCl. This effect was not simply the result of HU-210 interfering with the association between saccharin and LiCl because neither LiCl-induced suppressed hedonic reactions nor CTA (which are not selectively produced by nausea, see Grant, 1987; Parker *et al.*, 2008) were attenuated by this pretreatment. Indeed, only the reaction of gaping that is specifically produced by the nausea induced by LiCl was blocked by pretreatment with HU-210. Specifically, microinfusion of HU-210 into the VIC selectively attenuated the nausea pro-

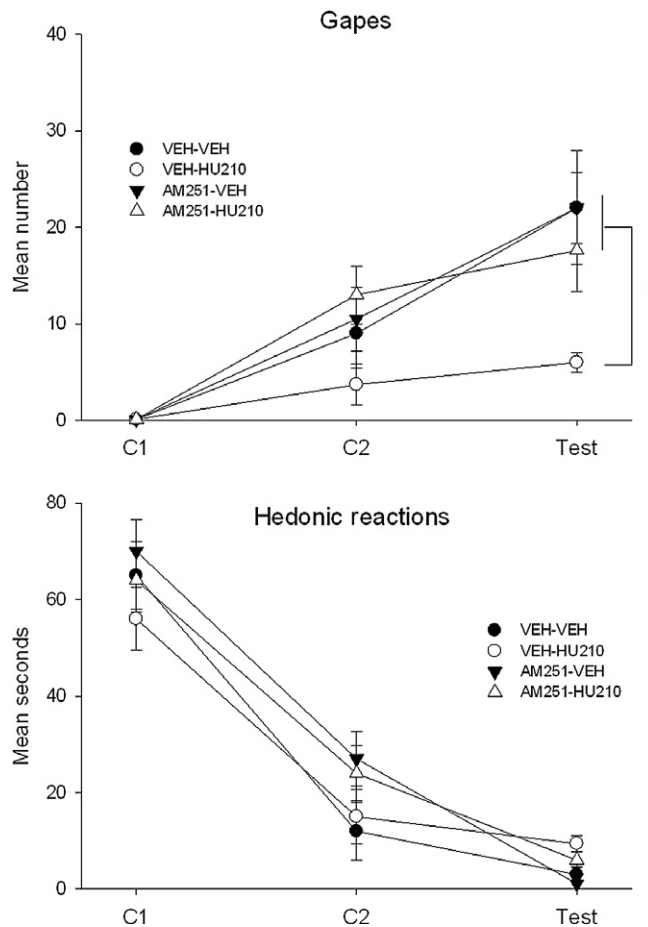


Figure 6

The mean ($\pm$ SEM) number of gaping responses (upper graph) and duration (s) of hedonic reactions (lower graph) expressed during the conditioning and test trials following microinfusion of the CB₁ antagonist/inverse agonist, AM-251, and the CB₁ agonist, HU-210, to the VIC. HU-210 attenuated LiCl-induced conditioned gaping reactions ($*P < 0.05$) and this effect was blocked by pretreatment with AM-251. No pretreatment altered hedonic reactions during the first conditioning trial or attenuated suppressed hedonic reactions during the second conditioning or test trials.

duced by LiCl, an effect that was reversed by AM-251 and is therefore mediated by CB₁ receptors.

The finding that the peripherally restricted compound CB13 interfered with conditioned gaping when delivered i.c.v., but not when administered systemically, confirms that the reduction of LiCl-induced nausea was centrally regulated. Indeed, at a dose of 3 mg·kg⁻¹, the centrally active THC, but not the peripherally restricted CB13, suppressed conditioned gaping. However, the finding that AM-251 did not produce nausea on its own when infused directly into the VIC was puzzling. Nevertheless, this latter finding is consistent with our recent report that systemic administration of AM-251 potentiates LiCl-induced nausea, but i.c.v. administration of AM-251 (at a dose as high as 1/10 the effective systemic dose) does not enhance LiCl-induced gaping (Limebeer *et al.*, 2010). Apparently, the production of nausea by CB₁ inverse agonists (Parker *et al.*, 2003; McLaughlin *et al.*, 2005)

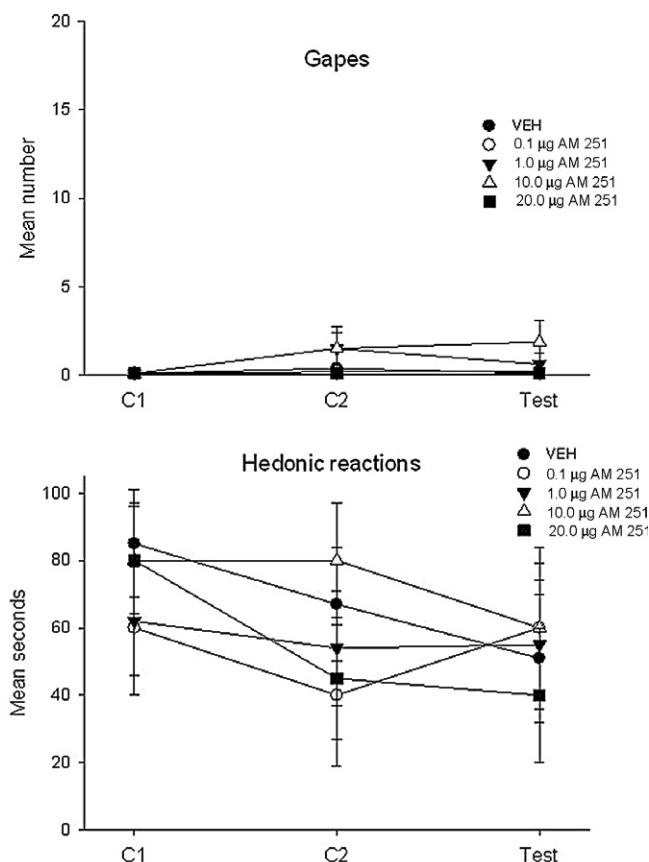


Figure 7

The mean ($\pm$ SEM) number of gaping responses (upper graph) and duration (s) of hedonic reactions (lower graph) expressed during the conditioning and test trials following microinfusion of AM-251 to the VIC subsequent to intraoral infusion of saccharin. There was no effect of drug treatment.

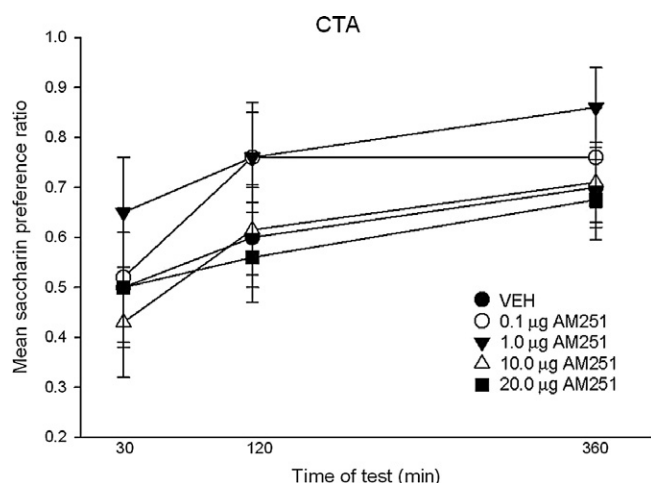


Figure 8

The mean ($\pm$ SEM) saccharin preference ratio expressed by the groups during the two-bottle test at 30, 120 and 360 min. There were no group differences.

originates peripherally. Interestingly, however, newly developed CB₁ receptor antagonists devoid of inverse agonist effects, including AM4113 (Sink *et al.*, 2008), AM6527 (Limebeer *et al.*, 2010) and AM6545 (Cluny *et al.*, 2010; Limebeer *et al.*, 2010) neither produce conditioned gaping reactions nor potentiate LiCl-induced conditioned gaping, suggesting that the nausea produced by SR141716 (Parker *et al.*, 2003) and AM-251 (McLaughlin *et al.*, 2005; Limebeer *et al.*, 2010) is mediated by inverse agonism of the CB₁ receptor. Other investigators have reported that CB₁ agonists decrease intestinal motility via peripheral CB₁ receptors (e.g. Pertwee, 2001), but act either centrally in the nucleus of the solitary tract in the brainstem (Van Sickle *et al.*, 2001; 2003) or peripherally (Darmani and Johnson, 2004) to attenuate emesis.

The IC receives visceral input in the granular region, VIC, and gustatory input in the adjacent dorsal agranular region (GIC; e.g. Lundy and Norgren, 2004; Saper, 2004). Therefore, the IC is an important site for taste-illness associations, which may lead to conditioned disgust reactions. Both electrolytic and neurochemical lesions of the GIC have been shown to interfere with the establishment of LiCl-induced CTA (e.g. Braun *et al.*, 1972; Bermudez-Rattoni and McGaugh, 1991; Cubero *et al.*, 1999; Roman and Reilly, 2007). Lesions that fall within the VIC do not interfere with LiCl-induced CTA (Mackey *et al.*, 1986; Yamamoto, 1993; Yamamoto *et al.*, 1995; Gedes *et al.*, 2008) even though inactivation of the VIC attenuates LiCl-induced malaise (Contreras *et al.*, 2007), as measured by them lying flat on their stomachs (Parker *et al.*, 1984; Bernstein *et al.*, 1992), which is also attenuated by area postrema lesions (Bernstein *et al.*, 1992). Using the conditioned disgust reaction measure, Kiefer and Orr (1992) found that ablation of the entire IC eliminated the establishment of LiCl-induced conditioned disgust reactions (without modifying the reaction to bitter quinine), but not taste avoidance. Our results suggest that the region of importance in these ablations was the VIC.

Penfield and Faulk (1955) observed that electrical stimulation of the insula in conscious patients undergoing surgery produced sensations of nausea. In addition, functional MRI research has demonstrated that the insula is engaged when a human views pictures of disgusting food (Calder *et al.*, 2007) or is exposed to disgusting smells (Heining *et al.*, 2003; Wicker *et al.*, 2003). Interestingly, the amygdala does not appear to be involved in processing conditioned disgust reactions in rats (Rana and Parker, 2008) or disgust reactions in humans (Calder *et al.*, 2007); instead, it appears to be more selectively activated in response to fear-evoking stimuli in humans (Calder *et al.*, 2007) and, in the case of the basolateral amygdala, CTA in rats (for review, see Reilly and Bornova, 2005), which may reflect conditioned fear rather than conditioned nausea (Parker *et al.*, 2008). Forebrain regions necessary for the production of the conditioned disgust reactions in rats are also necessary for the generation of nausea.

The establishment of these nausea-induced conditioned gaping reactions relies on an intact forebrain (Grill and Norgren, 1978b); that is, decerebrate rats display unconditional gaping reactions to a bitter taste, but do not display conditioned gaping reactions when exposed to a taste previously paired with a nausea-inducing treatment. Forebrain 5-HT may play a role in the generation of nausea because

lesions of the dorsal and median raphe, that reduce the amount of 5-HT available in the forebrain, interfere with the establishment of LiCl-induced conditioned gaping, but not taste avoidance (Limebeer *et al.*, 2004). Most recently, it has been demonstrated that selective 5,7-DHT lesions of the insular cortex that reduced 5-HT levels by 76% dramatically interfered with LiCl-induced conditioned gaping reactions (Tuerke *et al.*, 2010). Further, the classical anti-emetic agent, ondansetron, which attenuates LiCl-induced conditioned gaping reactions (and malaise-induced lying-on the belly; Tuerke *et al.*, 2011) when administered systemically (Limebeer and Parker, 2000), also attenuated these reactions when microinfused directly into the VIC, but not the GIC (Tuerke *et al.*, 2010). Therefore, the effects of ondansetron and HU-210 reported here on conditioned gaping reactions are equivalent. Although CB₁ agonists have been demonstrated to inhibit the function of 5-HT₃ receptors (Fan, 1995; Barann *et al.*, 2002; Racz *et al.*, 2008; Xiong *et al.*, 2011) as does ondansetron, it is unlikely that such a mechanism is responsible for the results reported here because the reduction of nausea by HU-210 was reversed by AM-251, suggesting a CB₁ mechanism. It is, however, likely that the endocannabinoid system may modulate the release of 5-HT in the VIC to regulate nausea in the normal functioning system. Indeed, considerable evidence points to an interaction between these two systems (Morales and Bloom, 1997; Hermann *et al.*, 2002; Morales and Bäckman, 2002; Morales *et al.*, 2004; Häring *et al.*, 2007). Future research is needed to elucidate how these interactions within the VIC may serve to regulate nausea.

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Conflict of interest

There is no conflict of interest on behalf of all the authors.

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