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Endocannabinoid regulation of nausea is mediated by 2-arachidonoylglycerol (2-AG) in the rat visceral insular cortex

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Abstract

Cannabinoid (CB) agonists suppress nausea in humans and animal models; yet, their underlying neural substrates remain largely unknown. Evidence suggests that the visceral insular cortex (VIC) plays a critical role in nausea. Given the expression of CB₁ receptors and the presence of endocannabinoids in this brain region, we hypothesized that the VIC endocannabinoid system regulates nausea. In the present study, we assessed whether inhibiting the primary endocannabinoid hydrolytic enzymes in the VIC reduces acute lithium chloride (LiCl)-induced conditioned gaping, a rat model of nausea. We also quantified endocannabinoid levels during an episode of nausea, and assessed VIC neuronal activation using the marker, c-Fos. Local inhibition of monoacylglycerol lipase (MAGL), the main hydrolytic enzyme of 2-arachidonoylglycerol (2-AG), reduced acute nausea through a CB₁ receptor mechanism, whereas inhibition of fatty acid amide hydrolase (FAAH), the primary catabolic enzyme of anandamide (AEA), was without effect. Levels of 2-AG were also selectively elevated in the VIC during an episode of nausea. Inhibition of MAGL robustly increased 2-AG in the VIC, while FAAH inhibition had no effect on AEA. Finally, we demonstrated that inhibition of MAGL reduced VIC Fos immunoreactivity in response to LiCl treatment. Taken together, these findings provide compelling evidence that acute

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Disclosures

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nausea selectively increases 2-AG in the VIC, and suggests that 2-AG signaling within the VIC regulates nausea by reducing neuronal activity in this forebrain region.

Keywords

2-arachidonoylglycerol (2-AG); c-Fos; endocannabinoid; insular cortex; monoacylglycerol lipase (MAGL); nausea

Introduction

Although the central neural circuitry underlying the vomiting reflex is well established (Hornby, 2001; Andrews and Horn, 2006; Horn, 2008), the precise brain mechanisms underlying nausea are less clear. Two recent functional neuroimaging studies in humans explored the brain circuitry involved in nausea (Napadow et al., 2013; Sclocco et al., 2014), and revealed an important role for the insular cortex; specifically, a strong sensation of nausea resulted in sustained activation of the interoceptive insula, as well as limbic and sub-cortical regions. Importantly, autonomic activation, which is important for nausea perception (LaCount et al., 2011), appears to be modulated by the insular cortex (Sclocco et al. 2014). This work suggests that the insula is part of a broad network of brain areas mediating the experience of nausea in humans (Napadow et al. 2013), and may play a particularly critical role in regulating autonomic control in response to nausea-inducing stimuli (Sclocco et al. 2014).

An intact forebrain is also necessary for the establishment of conditioned gaping responses in rats (Grill and Norgren, 1978a), a well-established rodent model of nausea (Parker, 2003; Parker et al., 2015). The insular cortex (IC) is particularly critical in this response, as its ablation was found to selectively disrupt conditioned gaping behavior (Kiefer and Orr, 1992). The IC (Cechetto and Saper, 1987; Allen et al., 1991) is comprised of gustatory neurons occupying the dysgranular (and agranular) layers (gustatory insular cortex [GIC]; Kosar et al. 1986), and visceral neurons located in the posterior granular layer (visceral insular cortex [VIC]; (Cechetto and Saper, 1987). We have previously shown that localized administration of the anti-emetic drug, ondansetron (a 5-hydroxytryptamine 3 [5-HT₃] antagonist), selectively blocked conditioned gaping upon intra-VIC - but not GIC - administration in rats (Tuerke et al. 2012a). Conversely, intra-VIC administration of a 5-HT₃ agonist, potentiated conditioned gaping caused by the emetic agent lithium chloride (LiCl), and even produced conditioned gaping on its own (Tuerke et al. 2012a). Moreover, Contreras et al. (2007) reported that administration of LiCl produced enhanced Fos expression in the VIC, and inactivation of this region attenuated LiCl-induced lying-on-belly behavior (lying with flattened belly on cage [LOB]), a measure of unconditioned nausea in rats (Parker et al. 1984; Tuerke et al. 2012b). Collectively, these studies demonstrate the importance of the IC, and particularly the VIC, in mediating behaviors reflective of the sensation of nausea in rats.

It is well known that exogenous cannabinoids exert robust anti-nausea and anti-emetic effects (Sharkey et al., 2014). We have shown that systemically administered cannabinoid (CB) agonists (Limebeer and Parker, 1999; Parker and Mechoulam, 2003; Parker et al.,

2003) or intra-cerebral administration of the synthetic cannabinoid agonist HU-210 into the VIC (Limebeer et al. 2012) reduced nausea-induced conditioned gaping in rats. Furthermore, exogenous administration of the endocannabinoid 2-arachidonoylglycerol (2-AG; Mechoulam et al. 1995; Sugiura et al. 1995), but not anandamide (AEA; Devane et al. 1992) within the VIC suppressed nausea-induced conditioned gaping, as well (Sticht et al., 2015). Surprisingly, this effect of 2-AG was not mediated via CB₁ receptors, but rather dependent on endocannabinoid metabolic products (Sticht et al. 2015). The physiologic role of endogenously released endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the VIC during nausea is still unknown, however; thus, the role of the endocannabinoid system in regulating this sensation remains to be determined.

We hypothesize that the VIC endocannabinoid system is recruited during an episode of nausea, and as such, regulates this sensation in rats. The rapid hydrolysis of endocannabinoids in vivo represents a significant challenge in investigating the physiological functions of these lipids; therefore, we investigated the role of endocannabinoids in the VIC via localized inhibition of their catabolic enzymes. This approach has been effectively used to elucidate the physiological role of the endocannabinoid system in several pre-clinical models, including pain (Davis, 2014), anxiety and depression (Gaetani et al., 2009), addiction and withdrawal (Sidhpura and Parsons, 2011; Muldoon et al., 2013), and nausea (Parker et al., 2014). Specifically, drugs that block their respective catabolic enzymes, fatty acid amide hydrolase (FAAH; Cravatt et al. 1996) and monoacylglycerol lipase (MAGL; Dinh et al. 2002) produce elevated brain levels of 2-AG and AEA. Additionally, we quantified endocannabinoid levels in the VIC during an episode of nausea to infer whether this system plays a tonic role in the regulation of nausea, an effect that is achieved through endocannabinoid (and CB₁)-mediated inhibition of neuronal signaling in this region. To this end, given the role for VIC activation during an episode of nausea (Contreras et al., 2007), we assessed whether the parameters of LiCl administration in the current study leads to elevated c-Fos within the VIC, and whether the anti-nausea effects following MAGL inhibition are associated with a subsequent decrease in neuronal activity in this region.

Methods

Animals

Sprague-Dawley rats (300–350 g; Charles River Lab, St Constant, Quebec) were single-housed in opaque plastic cages (10.25"W× 18.75"D ×8"H; 22 °C) with Teklad corn cob bedding (Harlan). Shredded paper and opaque tube/container were placed in each cage for environmental enrichment. Rats were maintained on a reverse light/dark cycle (7:00 am lights off; 7:00 pm lights on) with free access to food (Iams rodent chow, 18% protein). Behavioral testing occurred during the dark cycle; thus, nausea-induced behavior was assessed during the animals' wakeful state. All experiments were approved by the Animal Care Committee of the University of Guelph and carried out in accordance with the recommendations of the Canadian Council on Animal Care.

Drugs

The pre-treatment drugs were administered in a vehicle solution consisting of Tween 80 (Sigma Aldrich) and saline in a 1:9 ratio. Specifically, the compounds were first dissolved in ethanol and Tween 80, and the ethanol was subsequently evaporated off with a nitrogen stream. Saline was then added and the solution was delivered bilaterally into the VIC in a total volume of 1 μ l (Sticht et al., 2015). The dual FAAH/MAGL inhibitor, JZL195 (Cayman Chemical, Ann Arbor, MI, USA), was prepared at a concentration of 10 μ g/ μ l (Long et al., 2009). The selective FAAH inhibitor, URB597 (Cayman Chemicals), was prepared at a concentration of 0.01 μ g/ μ l, while the selective FAAH inhibitor, PF3845 (provided by B.F. Cravatt), was prepared at a concentration of 2 μ g/ μ l. The selective MAGL inhibitor, MJN110 (provided by B.F. Cravatt), was prepared at a concentration of 2 μ g/ μ l. The selective CB₁ antagonist/inverse agonist, AM251 (Cayman Chemicals), was prepared at a concentration of 2 μ g/ μ l. The non-selective cyclooxygenase (COX) inhibitor, indomethacin (Sigma Aldrich, St. Louis, MO, USA), was prepared at a concentration of 1 μ g/1 μ l. The drug doses were selected on the basis of previously established findings, where possible; for example, 0.01 μ g URB597 has been widely used for intra-cerebral administration (e.g. Rubino et al., 2008; McLaughlin et al., 2012) and was, therefore, used in the current study. However, the novel FAAH and/or MAGL inhibitors have not been previously administered into the brain. As such, a pilot experiment determined the effectiveness of 10 μ g JZL195 to reduce nausea following intra-VIC administration. For MJN110 and PF3845, a maximally effective dosage (i.e. 10 mg/kg) for systemic administration (Parker et al., 2014; Rock et al., 2015) was converted to total drug dose (approximately ~4 mg/rat), and half of the equivalent systemically injected amount was diluted by three orders of magnitude (i.e. mg to μ g) and delivered into the VIC in the current study. Doses of AM-251 and indomethacin were chosen based on their ability to reverse the suppressive effects of 2-AG and the synthetic cannabinoid, HU-210, respectively (Limebeer et al., 2012; Sticht et al., 2015). The illness-inducing drug, LiCl (0.15 M; Sigma Aldrich), was prepared in sterile water and administered (i.p.) at a volume of 20 ml/kg (127 mg/kg); this doses was selected on the basis of its effectiveness to produce conditioned gaping in rats (Limebeer and Parker, 2003).

Stereotaxic and intraoral (IO) cannulation surgery

The rats were allowed six days to habituate to the facility and then underwent surgical implantation of intracranial guide cannulae and IO cannulae according to previously described methods (Limebeer et al., 2012; Sticht et al., 2015). Under isoflurane anesthesia, the animals were implanted bilaterally with a 22 gauge cannulae directed at the VIC (at 10 degrees divergent angle; relative to Bregma: AP – 0.5; LM + 5.0; from the skull surface: DV – 4.5), and implanted with a 10-cm section of polyethylene tubing (PE 90) that extended subcutaneously from inside the oral cavity, exiting at the rear of the neck. The rats were allowed a two-week recovery period.

Behavioral procedures: Taste reactivity (TR) and conditioned taste avoidance (CTA)

All experiments consisted of a single adaptation session (3 min IO infusion of water), two conditioning trials (3 min IO infusion of saccharin; i.p. injection of LiCl) and a TR test (3

min IO infusion of saccharin), as previously described (Sticht et al., 2015). The TR test assesses responses to gustatory (flavor) stimuli infused directly into the oral cavity of a freely moving animal (Grill and Norgren, 1978b). Unlike an appetitive fluid consumption test of CTA, the TR test does not require an animal to initiate and maintain drinking behavior to assess acceptance or rejection of a taste (Grill and Norgren, 1978b). Moreover, only emetic/nausea-inducing drugs result in conditioned gaping, whereas CTA is not limited to nausea-specific manipulations to produce avoidance of a drug-paired flavor, as well as the finding that anti-emetic drugs selectively reduce conditioned gaping, but not CTA. As such, aversive gaping to intraorally infused LiCl-paired saccharin reflects a rejection of the nausea-paired taste (Garcia et al., 1974) and is a more selective measure of nausea-induced behavior (Parker, 2003).

Effect of intra-VIC dual FAAH/MAGL inhibition on LiCl-induced conditioned gaping—The rats were individually placed into a clear plexiglass TR chamber (29 × 29 × 10 cm; resting atop a clear glass plate) for one min prior to receiving a three min IO infusion of saccharin (0.1%) via an infusion pump. They were then removed and received bilateral VIC infusions of vehicle ($n = 7$) or JZL195 (10 µg, $n = 7$) at a rate of 0.5 µl/min for two min. The infuser was left in place for an additional min to allow the solution to diffuse into the tissue. Animals were subsequently placed back in their home cage prior to receiving an i.p. injection of LiCl (0.15M) 15 min later. Seventy-two hr following the second conditioning trial, rats underwent a TR test to assess gaping (wide mouth opening with bottom incisors exposed). We also sought to determine whether a lower dose of JZL195 reduces conditioned gaping; therefore, an additional cohort of animals received bilateral infusions of vehicle ($n = 7$) or 1 µg JZL195 ($n = 10$) following saccharin (15 min before LiCl), while a separate set of animals received the same dose of JZL195 ($n = 7$) but at a longer pre-treatment interval (70 min before LiCl) to allow greater accumulation of endocannabinoid levels.

To assess whether the drug manipulation selectively interfered with nausea – as opposed to a general interference with learning (conditioning) – we assessed the potential for JZL195 to modify CTA of LiCl-paired saccharin in a 2-bottle consumption test. Previous studies have shown that CTA and conditioned gaping are mediated by different processes; whereas conditioned gaping is selectively produced following nausea-inducing treatments, nausea is not a requirement for CTA (Parker, 2003). Following the TR test, rats were water deprived for 18 hr. They then received a two-bottle consumption test over a six hr period in which they were given free access to saccharin or water. A saccharin preference ratio was calculated as follows: total saccharin consumed/[total saccharin + total water].

Cannulae placements and dye spread analyses were verified as described previously (Sticht et al., 2015). The TR test videos were scored by an experienced observer blind to the experimental conditions using *The Observer* (Noldus Information Technology Inc., Leesburg, VA, USA), and with a high degree of interrater reliability ($r > .95$).

Effect of intra-VIC FAAH inhibition on LiCl-induced conditioned gaping—The procedure for this experiment was identical to the previous experiment with the exception that during conditioning rats received bilateral VIC infusions of URB597 at a dose consistent with those reported in the literature (0.01 µg; Rubino et al. 2008; McLaughlin et

al. 2012; Morena et al. 2014). We additionally assessed whether FAAH inhibition with URB597 and the novel inhibitor, PF3845 (2 µg; Ahn et al. 2009), would be more effective following a longer pre-treatment time period (70 min before LiCl) to allow greater accumulation of endogenous AEA. The rats were infused bilaterally with vehicle ($n = 13$), URB597 (15min, $n = 10$; 70min, $n=9$) or PF3845 ($n = 10$).

To determine whether FAAH inhibition would result in greater anti-nausea effects when combined with exogenous AEA, a second experiment assessed a set of animals that received either vehicle ($n = 13$) or URB597 (0.01 µg, $n = 6$; 70 min prior to LiCl) combined with AEA (0.4 µg; 15 min prior to LiCl). As before, the rats received a two-bottle consumption test 18 hr following the TR test.

Effect of intra-VIC MAGL inhibition on LiCl-induced conditioned gaping—We next assessed whether selective MAGL inhibition in the VIC would reduce nausea. The procedures for these experiments were identical to those above, however, during conditioning the rats received bilateral VIC infusions of vehicle ($n = 13$) or MJN110 (0.5 µg, $n = 5$; 2 µg, $n = 11$), which was administered 70 min prior to LiCl. To assess the mechanism by which MJN110 suppressed conditioned gaping, a separate group of animals was infused with MJN110 (2 µg, $n=11$), as well as with the CB₁ antagonist, AM251 (1µg, $n=11$), immediately before IO saccharin. We also assessed whether the cyclooxygenase inhibitor, indomethacin, would modify the suppressive effects of MJN110, as we have previously demonstrated a COX-dependent mechanism of action for 2-AG in suppressing nausea (Sticht et al., 2012, 2015). Therefore, following intra-VIC MJN110 (2 µg, $n=11$), a separate set of animals received indomethacin (0.5 µg, $n=6$) in the VIC immediately before IO saccharin.

Biochemical analyses

Quantification of VIC endocannabinoids following LiCl-induced nausea—Drug naïve rats were sacrificed 20 min following a systemic injection of 20 ml/kg of 0.15 M LiCl ($n= 5$) or saline ($n=5$) to assess endocannabinoid levels during an experience of acute nausea. This dose of LiCl reliably produces conditioned gaping reactions in rats (Grill & Norgren, 1978) and the time period was selected on the basis of reports that LiCl produces maximal malaise within 20 min of administration (Contreras et al. 2007; Parker et al. 1984). Rats were euthanized by rapid decapitation (restrained in a decapicone – Braintree Scientific, MA, USA), and their brains were immediately extracted; the VIC was subsequently dissected on a stainless steel platform resting atop dry ice, which resulted in rapid freezing of the tissue section. Tissue samples were stored at -80°C until the time of processing, which was performed at Virginia Commonwealth University.

Lipid extractions were carried-out according to previously described methods (Ramesh et al., 2011; Kinsey et al., 2013). Pre-weighed VIC samples were homogenized in 1.4 ml of buffer containing a 2:1 v/v chloroform:methanol solution (containing 0.0348 g phenylmethylsulfonyl fluoride/ml) after the addition of internal standards (4 pmol AEA-d8, 1 nmol 2-AG-d8, 330 pmol PEA-d4, 300 pmol OEA-d4, and 1 nmol AA-d8) to each sample. The homogenates were then mixed with 0.3 ml saline (0.73 % w/v) and subsequently

vortexed for one min. Following centrifugation (10 min at 3,220×g; 4°C), the aqueous phase and debris were separated and extracted another two times, each with 0.8 ml of chloroform; the organic phase from each of the separations was pooled together and the organic solvents were evaporated under a nitrogen stream (15 psi). After the samples were completely dried, they were reconstituted with 0.1 ml chloroform and, after vortexing, were mixed with 1 ml of cold acetone. After final centrifugation (5 min at 1,811×g;), the upper layer from each sample was collected and evaporated under nitrogen. The final dried constituents were reconstituted in 0.1 ml methanol and transferred to autosample vials for analysis.

AEA, 2-AG, OEA, PEA, and AA were quantified using LC/MS/MS. The mobile phase consisted of methanol: water (90:10) with ammonium acetate and 0.1 % formic acid. The column used was a Discovery® HS C18, 2.1×150 mm, 3 µm (Supelco, USA). Ions were analyzed in multiple reaction monitoring mode and the following transitions were monitored in positive mode: (348>62) and (348>91) for AEA; (356>62) for AEAd8; (379>287) and (379>269) for 2-AG; (387>96) for 2-AGd8; (300>62) and (300>283) for PEA; (304>62) for PEA-d4; (326>62) and (326>309) for OEA; and (330>66) for OEAd4; in negative mode: (303>259) and (303>59) for AA and (311>267) for AA-d8. A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.156 to 2.5 pmol for AEA, from 0.25 to 4 nmol for 2-AG, from 7.8 to 125 pmol for PEA and OEA, and from 1 to 16 nmol for AA.

Quantification of VIC endocannabinoids following intra-VIC catabolic enzyme inhibitor administration—Drug naïve rats were sacrificed 60 min following an intra-VIC infusion of vehicle ($n=5$), MJN110 (2 µg/µl; $n=7$), URB597 (0.01 µg/µl; $n=6$), or PF3845 (2 µg/µl; $n=7$). These doses and pre-treatment times were based on those used in the behavioral experiments in the present study. The VIC was subsequently extracted identical to that in the previous experiment and endocannabinoid levels were analyzed as described above.

Quantification of VIC endocannabinoids following systemically administered catabolic enzyme inhibitors—Drug naïve rats were sacrificed 120 min following an i.p. injection of vehicle ($n=9$), MJN110 (20 mg/kg; $n=6$), URB597 (0.3 mg/kg; $n=5$), or PF3845 (20 mg/kg; $n=6$). These doses and pre-treatment times were based on those used in prior behavioral studies demonstrating interference with LiCl-induced nausea (Cross-Mellor et al. 2007; Parker et al. 2014). The VIC was subsequently extracted and endocannabinoid levels were analyzed as described above.

Immunohistochemistry

Effect of MJN110 on LiCl-induced c-Fos immunoreactivity in VIC—Drug naïve rats were used to assess LiCl-induced c-Fos expression in the VIC. Rats first received daily handling and saline injections (20 ml/kg) for six days to habituate animals to the procedure. On the day of the experiment animals received a systemic (i.p.) injection of MJN110 (10 mg/kg) or vehicle 2 hr prior to a 20 ml/kg injection of LiCl (0.15 M) or saline ($ns = 8$ /group). Sixty minutes later, the animals were deeply anaesthetized with 1.5 ml sodium pentobarbital (340 mg/ml) and perfused intracardially with 300 ml of 4% paraformaldehyde

in phosphate buffered saline (PBS; pH 7.3); this time period was selected on the basis of a previous study (Contreras et al., 2007) and preliminary work, demonstrating robust increases in c-Fos expression 60 min after LiCl administration. The brains were subsequently extracted and postfixed in 4% paraformaldehyde at 4°C overnight, and then cryoprotected with 20% sucrose in PBS at 4°C overnight. With reference to a rat brain atlas (Paxinos and Watson, 2007), specimens were embedded in OCT compound (Tissue-Tek, Sakura Finetek Japan, Tokyo, Japan) and sectioned in the coronal plane (40 µm) on a cryostat. Floating sections at the coordinates described above containing the VIC were collected.

Coronal sections were washed three times (10 min intervals) in a solution composed of PBS and 0.1% Triton X-100, and then incubated in PBS blocking buffer containing 10% normal donkey serum for one hr at room temperature. Sections were incubated in primary antibody rabbit anti-c-Fos (1:2000; Oncogene, Cambridge, MA, USA) at 4°C for 48 hr. Tissue specimens were then washed in PBS containing 0.1% Triton X-100 three times for 10 min and incubated in donkey anti-rabbit CY3 (1:100; no. 711-166-152, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 2 hr. Sections were subsequently mounted in phosphate-buffered glycerol and examined using a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany). The gray-scale images were captured by digital camera (Qimaging, Surrey, BC, Canada). The mean number of c-Fos-immunoreactive nuclei was analyzed from the region of the VIC in which the pharmacological manipulations and eCB quantification occurred in the current study. Therefore, c-Fos counts were confined to a localized area of the VIC (600 × 450µm area) with a minimum of two sections/hemisphere used for each animal. Thus, there was a minimum of four sections per animal (n=4 per group). All images for the same region were obtained under the same exposure and magnification (10x) in all cases.

Data analysis

The frequency of gaping during the TR test in each experiment was analyzed using an analysis of variance (ANOVA) or *t*-tests, as appropriate, using SPSS Statistics version 22 (Armonk, NY, USA). The effect of JZL195 on LiCl-induced gaping was analyzed using an independent samples *t*-test (vehicle or 10 µg JZL195), or a one-way ANOVA with pre-treatment (vehicle, 1 µg JZL195 [15 min, 70 min]) as a between subjects factor. The effect of FAAH inhibition on gaping was assessed using a one-way ANOVA with pre-treatment (vehicle, PF3845, URB597 [15 min, 70 min]) as a between subjects factor. The effect of MAGL inhibition was assessed using a one-way ANOVA with pre-treatment (vehicle, MJN110 [0.5 µg, 2.0 µg]) as a between subjects factor. Separate *t*-tests were performed to assess combined URB597/AEA treatment on gaping and the effects of a CB₁ antagonist (AM-251) or COX inhibitor (indomethacin) on the suppressive effects of MJN110. An identical set of analyses was performed for the CTA data as described above.

The effect of LiCl on eCB (and PEA/OEA) levels in the VIC and cerebellum was analyzed using separate independent *t*-tests (saline, LiCl). The effect of eCB catabolic enzyme inhibitors on VIC 2-AG/AEA (and PEA/OEA) levels was analyzed using one-way ANOVAs with pre-treatment (vehicle, MJN110, PF3845, URB597) as a between subjects factor. The effect of eCB catabolic enzyme inhibitors on VIC c-Fos was analyzed using a

one-way ANOVA with pre-treatment (vehicle-saline, vehicle-LiCl, MJN110-saline, MJN110-LiCl) as a between subjects factor. As appropriate, Bonferonni *post hoc* tests were conducted. Significance for all analyses was set at $p < 0.05$.

Results

Intra-VIC administration of the dual FAAH/MAGL JZL195 reduces nausea-induced conditioned gaping

The dual FAAH/MAGL inhibitor JZL195 suppressed LiCl-induced conditioned gaping reactions. Figure 1A shows that rats receiving intra-VIC infusions of JZL195 prior to LiCl treatment displayed fewer gapes to LiCl-paired saccharin ($t(12)=2.48$, $p < 0.05$) during the TR test. To assess whether JZL195 selectively interfered with nausea – as opposed to a general interference with learning (conditioning) – we assessed whether this compound modified CTA of LiCl-paired saccharin in a two-bottle consumption test. The rats received a six hr preference test 24 hr following the TR test, in which they were given free access to saccharin or water. As seen in Figure 1A, JZL195 treatment did not reduce CTA to LiCl-paired saccharin ($t(12)=2.21$, $p < 0.05$); in fact, rats pre-treated with JZL195 consumed significantly less LiCl-paired saccharin. This pattern is consistent with previous findings in which intra-VIC 2-AG reduced conditioned gaping while increasing subsequent avoidance during a consumption test (Sticht et al., 2015). Lastly, a low dose of JZL195 (1 μ g) given 15 min or 70 min before LiCl did not reduce conditioned gaping reactions ($F(2,21)=0.40$, *ns*), as seen in Figure 1B. A representative photomicrograph of a VIC cannulae placement is presented in Figure 1C. All cannulae placements were located between a range from 0.00 and -1.32 mm posterior to Bregma (Figure 1D). Furthermore the dye spread analysis revealed a diffusion area within a range of 0.5–1.0 mm (mean = 0.65; SE= 0.04) A/P and 0.5–1.25 mm (mean =0.82; SE=0.6) M/L within the VIC.

Selective MAGL inhibition, but not FAAH inhibition, in the VIC reduces nausea-induced conditioned gaping

Given the effectiveness of dual FAAH/MAGL inhibition in the VIC to reduce nausea, we next assessed whether selective inhibitors of FAAH and MAGL within the VIC were sufficient to reduce conditioned gaping. As seen in Figure 2A, VIC infusion of either FAAH inhibitor did not affect conditioned gaping reactions. Specifically, neither PF3845 (2 μ g) nor URB597 (0.01 μ g; 15 min or 70 min prior to LiCl) infusion into the VIC had any effect on the establishment of conditioned gaping ($F(3,38)=1.85$, *ns*). These findings suggest that FAAH inhibition alone within the VIC was not sufficient to reduce the illness-inducing effects of LiCl. Therefore, we assessed whether URB597 would suppress nausea when combined with exogenous AEA administered immediately following IO saccharin. As seen in Figure 2B, concomitant pre-treatment with URB597 (0.01 μ g) and AEA (0.4 μ g) significantly reduced conditioned gaping during the TR test ($t(17)=2.39$, $p < 0.05$), without modifying avoidance of LiCl-paired saccharin during the two-bottle consumption test ($t(17)=0.46$, *ns*). Cannulae placements for all FAAH inhibition experiments are presented in Figure 2C.

We next examined whether selective MAGL inhibition in the VIC would reduce nausea. As seen in Figure 3A, bilateral intra-VIC infusions of MJN110 (0.5 μ g, 2 μ g) 70 min before the LiCl acquisition sessions suppressed the establishment of conditioned gaping, ($F(2,26)=5.23$, $p<0.05$), without affecting avoidance of LiCl-paired saccharin in the consumption test ($F(2,26)=0.63$, ns). To assess the mechanism by which MJN110 suppressed conditioned gaping, separate groups of animals were infused with the CB₁ antagonist AM251 (1 μ g) prior to MJN110. We also assessed whether the COX inhibitor, indomethacin, would modify the suppressive effects of MJN110, as we have previously demonstrated a COX-dependent mechanism of action for 2-AG in suppressing nausea (Sticht et al. 2012; Sticht et al. 2015). The suppressive effects of high dose MJN110 (2 μ g) were reversed following concomitant intra-VIC infusions of AM251 ($t(20)=-2.06$, $p=0.05$; Figure 3B), but not indomethacin ($t(15)=0.77$, $p=0.46$; Figure 3C). Cannulae placements for all MAGL inhibition experiments are presented in Figure 3D.

Acute LiCl-induced nausea selectively increases 2-AG within the VIC

We next investigated the impact of an acute nausea episode produced by acute LiCl treatment on AEA and 2-AG levels within the VIC. LiCl administration led to significant elevations in VIC 2-AG levels at 20 min compared with saline-treated controls ($t(8)=2.80$, $p<0.05$), while AEA levels were unchanged ($t(8)=1.22$, ns ; Figure 4A). There was also a significant reduction in OEA levels (Table 1) compared to animals injected with saline ($t(8)=2.99$, $p<0.05$), whereas levels of PEA remained unchanged. To evaluate whether the LiCl-induced increase in 2-AG was selective to the VIC, endocannabinoid levels in the cerebellum were assessed in a separate group of animals, a region not shown to be involved in pharmacologically-induced nausea or emesis. As seen in Figure 4B, AEA and 2-AG levels did not significantly differ between LiCl-treated rats and saline-treated control rats.

Inhibition of endocannabinoid catabolic enzymes increases 2-AG levels, but not AEA levels, in the VIC

In order to assess the effectiveness of endocannabinoid catabolic enzyme inhibitors in elevating AEA and 2-AG, we quantified endocannabinoid levels following localized MAGL/FAAH inhibition in the VIC. Intra-VIC administration of MJN110 (2 μ g) increased 2-AG levels, ($F(3,21)=5.64$, $p<0.01$; Figure 4C) but the FAAH inhibitors were without effect; specifically, in contrast to intra-VIC MJN110, URB597 (0.01 μ g) or PF3845 (2 μ g) failed to affect AEA levels ($F(3,21)=1.69$, ns ; Figures 4D). However, analysis of PEA ($F(3,21)=7.65$, $p=0.001$) and OEA ($F(3,21)=4.03$, $p<0.05$) levels in the VIC revealed significant effects of intra-VIC drug treatment (Table 1); PF3845 treated rats displayed significantly higher PEA levels compared to vehicle and MJN110 treated rats ($p<0.01$), as well as URB597 treated ($p<0.05$) animals. On the other hand, PF3845 treated rats only displayed significantly higher OEA levels relative to MJN110 treated rats ($p<0.05$).

We next assessed the potential for systemically administered enzyme inhibitors to increase 2-AG and AEA levels in the VIC. Analysis of VIC endocannabinoid levels following systemic enzyme inhibitor administration revealed selective increases in 2-AG ($F(3,22)=30.64$, $p<0.001$) following an injection of MJN110 (20 mg/kg, i.p.; Figure 4E); however, AEA levels were not elevated in response to systemically administered URB597

(0.3 mg/kg, i.p.) or PF3845 (20 mg/kg, i.p., $F(3,22)=1.07$, ns ; Figure 4F). As seen in Table 1, single-factor ANOVAs also revealed significant main effects of drug treatment on PEA ($F(3,22)=133$, $p<0.001$) and OEA ($F(3,22)=132$, $p<0.001$) levels in the VIC; subsequent post hoc tests revealed that URB597-treated rats displayed significantly greater OEA levels ($p<0.05$), while rats that received PF3845 displayed greater PEA and OEA levels relative to all other treatment groups ($p<0.001$).

MAGL inhibition reduces nausea-induced VIC neuronal activation

Given our findings above, we investigated whether the MAGL inhibitor, MJN110, reduces neuronal activation in response to acute LiCl-induced nausea. We first assessed whether the LiCl dose used in the current study (20 ml/kg of 0.15 M) resulted in a significant degree of neuronal activation. Systemic injection of LiCl resulted in substantial c-Fos expression relative to saline-treated control animals (data not shown). We subsequently assessed whether systemically administered MJN110 (10 mg/kg, i.p.; Parker et al. 2014) decreases neuronal c-Fos expression induced by LiCl. As seen in Figures 5A and 5B, LiCl increased c-Fos immunoreactivity, and this effect was prevented by pretreatment with MJN110 (10 mg/kg, ip). Vehicle and saline (pre-treatment/treatment) injections resulted in a higher basal c-Fos immunoreactivity in the VIC compared to the single vehicle injection (data not shown), however, c-Fos immunoreactivity was still significantly increased in animals treated with LiCl ($F(3,60)=4.79$, $p < 0.01$). Subsequent Bonferroni post hoc comparisons revealed that rats administered the vehicle-LiCl treatment had elevated c-Fos expression compared to those receiving vehicle-saline or MJN110-LiCl ($p < 0.05$). The MJN110-saline group did not significantly differ from any other group.

Discussion

The insular cortex is increasingly recognized as a critical forebrain region involved in the sensation of nausea (e.g., Contreras et al. 2007; Napadow et al. 2013; Sclocco et al. 2014). Here, we demonstrate that the endocannabinoid 2-AG serves as an endogenous regulator of nausea in the rat VIC, an effect mediated through CB₁ receptors. Indeed, 2-AG was found to be selectively elevated in the VIC during an episode of LiCl-induced nausea. Furthermore, LiCl-induced neuronal activation in this region was prevented by pre-treatment with the MAGL inhibitor, MJN110, which we have shown selectively elevates 2-AG in the VIC. These results, therefore, provide compelling evidence for nausea regulation by the VIC endocannabinoid system – an effect attributable to the endocannabinoid, 2-AG.

In the first set of experiments, the dual FAAH/MAGL inhibitor, JZL195, was found to suppress the establishment of conditioned gaping upon intra-VIC administration, suggesting an anti-nausea role for manipulations that elevate levels of AEA and 2-AG within this forebrain area. However, administration of the selective FAAH inhibitors, URB597 (Fegley et al., 2005) or PF3845 (Ahn et al., 2009), were ineffective in reducing conditioned gaping, suggesting that FAAH inhibition alone in the VIC is not sufficient to suppress nausea or elevate AEA levels. Indeed, the endocannabinoid analyses in the current study revealed that neither systemic nor intra-VIC administration of the FAAH inhibitors URB597 or PF3845, elevated AEA in the VIC, although PEA and OEA (other FAAH substrates) were elevated,

indicating that the doses were sufficient to suppress FAAH and increase fatty acid ethanolamides. In this case, PF3845 had a greater effect on PEA/OEA, which is consistent with Ahn et al. (2009).

The failure of FAAH inhibition to elevate AEA in the VIC is particularly interesting given that centrally administered URB597, with nearly identical dose/pre-treatment procedures, has been reported by others to elicit AEA-induced anxiolytic effects (Rubino et al., 2008), as well as stress-coping behavior through an AEA (and CB₁-dependent) mechanism (McLaughlin et al., 2012). We have previously reported that exogenous AEA administration in the VIC is also ineffective in reducing conditioned gaping (Sticht et al., 2015), and the current study further demonstrates the ineffectiveness of pro-AEA manipulations within the VIC to reduce nausea. Taken together, it appears that endogenously released AEA in the VIC may not play a physiological role in endocannabinoid-suppression of nausea. This observation is particularly surprising, because AEA levels are clearly increased in the brainstem and contributes to the anti-emetic effects of the endocannabinoid system in response to emetic stimuli (Van Sickle et al., 2005). Nevertheless, the current findings suggest that manipulations resulting in large increases in AEA levels (such as with exogenous administration and combined FAAH inhibition) may exert anti-nausea effects through an action in the VIC - but only under conditions of reduced AEA metabolic turnover.

On the other hand, MAGL inhibition alone reduced nausea, as intra-VIC infusions of MJN110 prevented the establishment of LiCl-induced conditioned gaping. This anti-nausea effect of MJN110 was subsequently blocked by the CB₁ antagonist, AM-251. Indeed, both systemic and intra-VIC administration of MJN110 significantly elevated 2-AG levels, but did not affect AEA or other fatty acid amides in the VIC. Presumably, MAGL inhibition enables 2-AG to directly activate CB₁ receptors for a sufficient period of time to reduce the nauseating effects of LiCl. Given that 2-AG is a rate limiting substrate of free arachidonic acid in the brain (Nomura et al., 2008, 2011), however, it is likely that metabolites of this eicosanoid represent an additional anti-nausea system, as well. In support of this view, we have previously shown that exogenous 2-AG delivered into the VIC reduces acute nausea through a CB₁ receptor independent mechanism of action (Sticht et al., 2015), and systemic administration of 2-AG also reduces nausea independent of CB₁ receptors (Sticht et al., 2012). Unlike in the current study, however, MAGL-mediated hydrolysis of 2-AG renders the anti-nausea effects of 2-AG at CB₁ receptors relatively short-lived; thus, downstream metabolic targets of 2-AG are particularly relevant under conditions of increased 2-AG turnover, and, therefore, explain why its anti-nausea effects can be blocked following COX inhibition.

The regulatory role of the endocannabinoid system in nausea was further investigated in the present study through the quantification of endocannabinoid levels in the VIC during an episode of LiCl-induced nausea. Twenty min following an i.p. injection of LiCl, the time of peak malaise (Parker et al. 1984; Contreras et al. 2007), 2-AG (but not AEA) was elevated in the VIC. Moreover, we assessed the effectiveness of the MAGL inhibitor, MJN110, to reduce VIC neuronal activation in response to acute LiCl-induced nausea. Indeed, LiCl enhanced the expression of c-Fos in the VIC (replicating Contreras et al. 2007, albeit at a

four-fold higher dose), and this effect was blocked by pretreatment with systemic MJN110 at a dose that suppressed the establishment of LiCl-induced conditioned gaping in rats (Parker et al. 2015). This finding provides strong evidence that the nauseating effects of LiCl were prevented by the suppression of VIC neural activation, achieved via increased 2-AG signaling at CB₁ receptors.

Evidence from our group suggests that the nauseating/activating effects of LiCl may be produced by 5-HT (Tuerke et al. 2012a; Limebeer et al. 2004). We previously demonstrated that a 76% reduction of 5-HT (by 5,7-DHT lesions) in the entire insular cortex dramatically suppressed the acquisition of conditioned gaping reactions elicited by a LiCl-paired flavor. Furthermore, in a double dissociation, we found that intra-VIC administration of the 5-HT₃ receptor antagonist, ondansetron, attenuated LiCl-induced conditioned gaping reactions, but not taste avoidance. Conversely, the direct delivery of a 5-HT₃ receptor agonist into these regions produced the opposite effect. Therefore, 5-HT₃ activity in the VIC, a site with gastrointestinal input, is critical for the establishment of nausea-induced conditioned gaping in rats (Tuerke et al. 2012a). The emetic drug, LiCl, has also been shown to elevate 5-HT in several brain areas (for up to one hr) following acute administration (Otero Losada and Rubio, 1986; West et al., 1991; Zach et al., 2006). Although it remains to be determined whether LiCl enhances 5-HT release within the VIC, the present study is, nonetheless, the first to quantify endocannabinoid levels in response to LiCl-induced nausea in rats, and represents an important step in understanding precisely how the endocannabinoid system modulates the sensation of nausea.

Given that VIC 5-HT signaling is critical for the establishment of LiCl-induced conditioned gaping (Tuerke et al. 2012a), it appears that 5-HT release in the VIC may underlie an experience of nausea, which is subsequently reduced by 2-AG-CB₁ signaling in this forebrain region. Considerable evidence supports the co-localization of CB₁ receptors on 5-HT presynaptic terminals in several brain regions (Barann et al., 2002; Hermann et al., 2002; Darmani and Johnson, 2004; Häring et al., 2007, 2013). Although it has not been determined if such co-localization occurs in the VIC, this scenario is likely given the CB₁-dependent effects of 2-AG in the current study, as well as our recent finding that the potent synthetic cannabinoid receptor agonist, HU-210, reduced LiCl-induced conditioned gaping when localized to the VIC (but not GIC). Furthermore, the suppression of nausea-induced behavior was reversed by pre-treatment with the CB₁ antagonist, AM251, indicating that the anti-nausea effects were CB₁-mediated. Future studies will directly assess the potential of MAGL inhibition to reverse LiCl-induced release of 5-HT in the VIC by employing an *in-vivo* microdialysis approach.

In conclusion, we have demonstrated that the endocannabinoid 2-AG is an endogenous regulator of nausea in the rat VIC. These findings extend previous observations from our group and others demonstrating that cannabinoids suppress nausea in animal models (Sharkey et al., 2014; Parker et al., 2015). It is also clear that the eCB system within the VIC serves an important modulatory function during an experience of nausea and that this role is sub-served by the multiple actions of 2-AG: During periods of reduced MAGL activity, the anti-nausea effects of 2-AG appear to be mediated primarily by CB₁ receptors, while metabolic (COX) products downstream of 2-AG also play an important role in mediating

some of its effects. Given that nausea results from numerous manipulations, pharmacologically-induced and otherwise, future studies must explore whether similar mechanisms (and yet to be uncovered ones) underlie nausea in response to a variety of illness-inducing treatments. Moreover, it also remains to be determined whether mechanisms beyond CB₁-signaling exist for the endocannabinoid system in the human experience of nausea, and the extent to which 2-AG regulates this sensation through an action in the human insular cortex. Indeed, further nausea-focused research is necessary to gain a more global understanding of the mechanisms underlying this debilitating sensation.

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Abbreviations

2-AG	2-arachidonoylglycerol
5-HT₃	5-hydroxytryptamine 3
AEA	anandamide
CB	cannabinoid
CTA	conditioned taste avoidance
COX	cyclooxygenase
FAAH	fatty acid amide hydrolase
GIV	gustatory insular cortex
IC	insular cortex
IO	intraoral
LiCl	lithium chloride
LOB	lying on belly
MAGL	monoacylglycerol lipase
OEA	oleoylethanolamide
OEA	palmitoylethanolamide
TR	taste reactivity
VIC	visceral insular cortex

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- Visceral insular cortex (VIC) 2-AG was increased during LiCl-induced nausea in rats
- Intra-VIC MAGL inhibition suppressed nausea-induced conditioned gaping
- The anti-nausea effects of MAGL inhibition were attenuated following CB₁ antagonism
- MAGL inhibition reduced LiCl-induced Fos immunoreactivity within the VIC
- The endocannabinoid system regulates nausea via 2-AG and CB₁ signaling in the VIC

Figure 1. Dual FAAH/MAGL inhibition in the VIC reduces nausea-induced gaping

Effect of localized dual FAAH/MAGL inhibition in the VIC on the establishment of LiCl-induced conditioned gaping and conditioned taste avoidance. **A.** Mean ($\pm$ SEM) number of gapes during the TR test and saccharin preference during the two-bottle test among rats that received intra-VIC infusions of the dual FAAH/MAGL inhibitor, JZL195 (10 µg; $n=7$) or vehicle ($n=7$) 15 min prior to LiCl. The 10 µg dose of JZL195 reduced LiCl-induced conditioned gaping ($p < 0.05$) and enhanced saccharin avoidance ($p < 0.05$). **B.** Mean ($\pm$ SEM) number of gapes during the TR test among rats that received intra-VIC infusions of vehicle ($n=13$) or 1 µg JZL195 15 min ($n=10$) or 70 min ($n=7$) prior to LiCl. The 1 µg dose of JZL195 did not affect conditioned gaping. **C.** Representative photomicrograph of VIC cannulation. **D.** Schematic representation of VIC cannula tip placements for all JZL195 and vehicle treated rats (Paxinos and Watson 2007).

Figure 2. Selective FAAH inhibition alone is ineffective in reducing nausea-induced gaping
 Effect of localized FAAH inhibition in the VIC on the establishment of LiCl-induced conditioned gaping and conditioned taste avoidance. **A.** Mean ($\pm$ SEM) number of gapes during the TR test among rats that received intra-VIC infusions of vehicle ($n=13$), or the FAAH inhibitors, URB597 (15 min, $n=10$; 70 min, $n=9$) and PF3845 (70 min, $n=10$) prior to LiCl. Pre-treatment with either FAAH inhibitor did not reduce conditioned gaping. **B.** Mean ($\pm$ SEM) number of gapes during the TR test and saccharin preference during the two-bottle test among rats that received intra-VIC infusions of 0.01 μ g URB597 combined with 0.4 μ g AEA ($n=6$) or vehicle alone ($n=13$) prior to LiCl. The combined URB597/AEA pre-treatment significantly reduced conditioned gaping ($p<0.05$) compared to vehicle treated rats, without modifying saccharin taste avoidance. **C.** Schematic representation of VIC cannula tip placements for all FAAH inhibition experiments (Paxinos and Watson, 2007).

Figure 3. Selective MAGL inhibition in the VIC reduces nausea-induced gaping

Effect of localized MAGL inhibition in the VIC on the establishment of LiCl-induced conditioned gaping and conditioned taste avoidance. **A.**, Mean ($\pm$ SEM) number of gapes during the TR test and saccharin preference during the two-bottle test (**B.**) among rats that received intra-VIC infusions of vehicle ($n=13$) or the selective MAGL inhibitor, MJN110 (0.5 μ g, $n=5$; 2 μ g, $n=11$). The highest dose of MJN110 (2 μ g) significantly reduced conditioned gaping ($p<0.05$) but did not modify conditioned taste avoidance. **C.**, The anti-nausea effects of 2 μ g MJN110 were reversed following pre-treatment with the CB₁ antagonist, AM-251 (1 μ g, $n=11$; $p = 0.05$), but not the cyclooxygenase inhibitor, indomethacin (0.5 μ g, $n=6$;). **D.** Schematic representation of VIC cannula tip placements for all MAGL inhibition experiments (Paxinos and Watson, 2007).

Figure 4. LiCl-induced nausea selectively increases 2-AG in the VIC

Effect of LiCl-induced nausea and intra-VIC or systemically administered catabolic enzyme inhibitors on VIC endocannabinoid levels. **A. & B.**, Mean ($\pm$ SEM) endocannabinoid levels within the VIC (**A**) and cerebellum (**B**) 20 min following an i.p. injection of nausea-inducing LiCl (0.15 M; 20 ml/kg; $n=5$) or saline ($n=5$). Intra-VIC content of 2-arachidonoylglycerol (2-AG), but not *N*-arachidonylethanolamide (AEA), was significantly greater ($p<0.05$) among rats that received LiCl compared to saline injected control animals, whereas no change in endocannabinoid levels was detected in the cerebellum. **C. & D.**, Mean ($\pm$ SEM) endocannabinoid levels within the VIC 70 min following an intra-VIC infusion of the MAGL inhibitor, MJN110 (2 μ g; $n=7$), or either of the FAAH inhibitors, URB597 (0.01 μ g; $n=6$) and PF3845 (2 μ g; $n=7$), or vehicle ($n=5$). Intra-VIC administration of MJN110

selectively increased 2-AG content (**C**; $p<0.05$) in the VIC, whereas AEA levels (**D**) remained unchanged following an injection of either URB597 or PF3845 relative to vehicle treated control rats. **E. & F.**, Mean ($\pm$ SEM) endocannabinoid levels within the VIC 120 min following an i.p. injection of the MAGL inhibitor, MJN110 (20 mg/kg; $n=6$), or either of the FAAH inhibitors, URB597 (0.3 mg/kg; $n=5$) and PF3845 (20 mg/kg; $n=6$), or vehicle ($n=9$). MJN110 selectively increased 2-AG content (**E**; $p<0.001$) in the VIC, whereas AEA levels (**F**) remained unchanged following an injection of either URB597 or PF3845 relative to vehicle control rats.

Figure 5. MAGL inhibition reduces LiCl-induced c-Fos in the VIC

Effect of systemic MAGL inhibition on LiCl-induced c-Fos activation in the VIC **A.** Mean ($\pm$ SEM) number of c-Fos immunoreactive cells within an area ($450 \times 600 \mu\text{m}^2$) of the VIC 3 hr following an i.p. injection of the MAGL inhibitor, MJN110 (10 mg/kg; $n=7$), or vehicle ($n=8$) and 1 hr following an injection of LiCl (0.15 M; 20 ml/kg; $n=7$) or saline (0.9 % NaCl; $n=8$). VIC c-Fos immunoreactivity was observed in all treatment groups; however, rats that received systemically administered vehicle with LiCl had significantly higher number of c-Fos immunoreactive cells compared to vehicle and saline treated control animals ($p < 0.05$), as well as those that received an i.p. injection of MJN110 and LiCl ($p < 0.05$). **B.** An immunofluorescence micrograph at 5X magnification illustrating the area of inspection within the VIC.

C. Representative immunofluorescence micrographs showing c-Fos immunoreactivity in the VIC under the various pre-treatment/treatment conditions. Scale bar = $50 \mu\text{m}$.

Table 1

Levels of the fatty acid ethanolamides, PEA and OEA, in the VIC following administration of nausea-inducing LiCl (20 min pre-treatment; $n=5$ /group), as well as intra-VIC (70 min pre-treatment; $n=5-7$ /group) or systemic (120 min pre-treatment; $n=5-9$ /group) endocannabinoid catabolic enzyme inhibitors, respectively. Values represent mean $\pm$ SEM.

Treatment	PEA (pmol/g)	OEA (pmol/g)
Lithium chloride (LiCl)		
Saline	206.3 $\pm$ 33.7	151.0 $\pm$ 11.8
LiCl	151.2 $\pm$ 12.9	93.4 $\pm$ 15.3*
Intra-VIC enzyme inhibitors		
Vehicle	589.3 $\pm$ 101.4	302.0 $\pm$ 53.7
MJN110	537.2 $\pm$ 71.1	276.2 $\pm$ 54.8
URB597	722.4 $\pm$ 122.4	355.4 $\pm$ 60.7
PF3845	1776.9 $\pm$ 361.3**	626.0 $\pm$ 121.3
Systemic enzyme inhibitors		
Vehicle	285.0 $\pm$ 38.2	113.2 $\pm$ 18.6
MJN110	224.9 $\pm$ 31.2	120.9 $\pm$ 24.0
URB597	682.0 $\pm$ 186.1	281.2 $\pm$ 6.8*
PF3845	2808.1 $\pm$ 156.3***	1049.5 $\pm$ 53.4***

PEA, palmitoylethanolamide; OEA, oleoylethanolamide;

 $p < 0.001$;

**
 $p < 0.01$;

*
 $p < 0.05$.