The MAGL Inhibitor, JZL184, Attenuates LiCl-Induced Vomiting in the *Suncus murinus* and 2AG Attenuates LiCl-Induced Nausea-Like Behavior in Rats

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ABSTRACT

THE MAGL INHIBITOR, JZL184, ATTENUATES LICL-INDUCED VOMITING IN THE *SUNCUS MURINUS* AND 2AG ATTENUATES LICL-INDUCED NAUSEA-LIKE BEHAVIOR IN RATS

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The role of 2-arachidonoylglycerol (2-AG) in nausea and vomiting was evaluated using a shrew (*Suncus murinus*) model of emesis and nausea-like behavior in rats, conditioned gaping. Shrews received JZL184, a selective MAGL inhibitor, prior to treatment with emetogenic lithium chloride (LiCl). The potential of exogenously administered 2-AG and arachidonic acid (AA) to regulate conditioned gaping was assessed in rats. The role of cannabinoid receptors and cyclooxygenase (COX) inhibition in suppression of vomiting and conditioned gaping was also evaluated. JZL184 dose-dependently suppressed vomiting in shrews, and was shown to inhibit MAGL in shrew brain tissue. The anti-emetic effects of JZL184 were prevented by the CB1 antagonist, AM251. Exogenous 2-AG suppressed LiCl-induced conditioned gaping, but was not prevented by AM251 or the CB2 antagonist, AM630. Instead, the COX inhibitor, indomethacin, prevented the suppressive effects of 2-AG, as well as AA. These results suggest that manipulations that elevate 2-AG may have anti-emetic/anti-nausea potential

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General Introduction

Cannabinoid drugs, both plant-based and synthetic derivatives, are treatment options for a broad range of disorders (Ligresti, Petrosino & Di Marzo, 2009). For example, Cannabinoid-containing medications provide effective relief for the anorexic side effects often accompanying treatment of hepatitis C virus (Costiniuk, Mills & Cooper, 2008), while other Cannabis-based drugs such as Sativex have been used to alleviate neuropathic pain and sleep disturbances caused by several different disorders (Perez & Ribera, 2008), and the synthetic cannabinoid, Nabilone, has been used to manage treatment-resistant nightmares among patients diagnosed with posttraumatic stress disorder (Fraser, 2009). Moreover, the therapeutic application of cannabinoid drugs has been proposed for the treatment of gastrointestinal, pancreatic and liver diseases (Izzo & Cammileri, 2008), and due to their potent immuno-modulatory properties, show promise in the development of novel treatments for various inflammatory disorders (Croxford & Yamamura, 2005). Conversely, drugs that block the biological action of cannabinoids also appear to have therapeutic potential, such that Rimonabant (Leite, Mocelin, Peterson, Leal & Thiesen, 2009) and Taranabant (Addy et al, 2008) have been used in the treatment of obesity. However, the earliest and most recognized medical use of cannabinoid has been for the management of nausea and vomiting (Parker & Limebeer, 2008).

Cannabinoids: Effects on Nausea and Vomiting in Humans

One of the most prevalent and recent therapeutic applications of cannabinoids is to alleviate chemotherapy-induced nausea and vomiting (CINV) among cancer patients. The experience of CINV is classified into distinct phases, based on the time of onset

from initial treatment (Bender et al, 2002): 1) Acute onset, which occurs within 24 hours or less of chemotherapy treatment. 2) Delayed onset, following the first day of initial treatment. 3) Anticipatory nausea and vomiting, which is a classically conditioned response to the environment that can occur within a number of months following treatment (Nesse et al, 1988) or prior to the administration of chemotherapy (Bender et al). When untreated by anti-emetics, CINV is experienced by approximately 75-80 percent of cancer patients (Schwartzberg, 2006; 2007), with patients reporting these as among the most distressing side effects of treatment (de Boer-Dennert et al, 1997). As these symptoms can significantly impair quality of life, it has been estimated that nearly 20 percent of patients decided to abandon treatment due to CINV (Jordan, Kasper & Schmoll, 2005).

Many drug treatments are accompanied by the unwanted side effects of nausea and vomiting within their effective dose ranges (Horn, 2008), which may impede both traditional and novel therapies due to potential dose-limiting toxicities (Andrews & Horn, 2006). The 5-hydroxytryptamine-3 (5-HT_3) receptor antagonists, such as ondansetron (OND), in combination with dexamethasone have been shown to provide effective relief of acute vomiting (Navari & Province, 2006), however, they are less effective in treating acute nausea (Andrews & Horn, 2006), and are ineffective in treating delayed or anticipatory nausea and vomiting (Aapro, 2005). Even greater than emesis, patients often consider nausea to be more problematic due to the longer duration of symptoms compared to the relatively brief episodes of vomiting (Andrews & Horn). Therefore, there is a need for more effective treatments of nausea, as well as delayed CINV and anticipatory nausea.

Evidence suggests that cannabinoid compounds are effective alternative treatments for CINV and anticipatory nausea (see Parker & Limebeer, 2008). Clinical trials throughout the 1970s and 1980s revealed the effectiveness of the Δ-9-tetrahydrocannabinol (THC) compound, Dronabinol (Marinol), and the synthetic compound, Nabilone, as anti-emetic agents (reviewed in Parker & Limebeer, 2008). Subsequent investigations found that Dronabinol and Nabilone provided superior anti-emetic effects compared to conventional non-cannabinoid antiemetics available at the time (Tramer et al. 2001), suggesting that cannabis-based medicines may be particularly effective in managing certain forms of treatment-resistant nausea and vomiting (Parker & Limebeer, 2008). Surprisingly, only one study has compared the effectiveness of THC with the more effective 5-HT antagonist, Ondansetron, in treating delayed chemotherapy-induced nausea and vomiting (Meiri et al. 2007); among patients receiving a moderately to highly emetogenic chemotherapy regimen, Dronabinol was found to be more effective than Ondansetron in reducing the intensity of nausea compared to a placebo treatment. Importantly, the dose of Dronabinol used in this study was at least 50 percent lower than in previous studies (e.g. Sallan et al. 1995; Sallan et al. 1998) resulting in a lower incidence of CNS-related adverse effects, which did not differ from incidence in the ondansetron treated group. Therefore, this finding supports the effectiveness of cannabinoids as an alternative treatment for CINV and delayed CINV, particularly among patients who do not respond to current antiemetic agents.

Anti-Emetic Effects of Cannabinoids in Animal Models

Animal models of nausea and vomiting function as pre-clinical tools to evaluate potential pharmacological treatments (Andrews & Horn, 2006), as well as provide an

opportunity to explore the neurobiology of nausea and emesis. THC, as well as synthetic cannabinoids, have been found to reduce or attenuate toxin-induced vomiting in cats (McCarthy & Borison, 1981), ferrets (Van Sickle et al. 2001; 2003), pigeons (Ferrari, Ottani & Giuliani, 1999), least shrews (*Cryptotis parva*; Darmani, 2001; 2002), and house musk shrews (*Suncus murinus*; Parker, Kwiatkowska, Burton & Mechoulam, 2003; Kwiatkowska, Parker, Burton & Mechoulam, 2004), often in a dose-dependent manner (see Parker & Limebeer, 2008). Parker et al. (2004) investigated the ability of THC and the non-psychoactive constituent of Cannabis, cannabidiol (CBD), to suppress vomiting induced by Lithium Chloride (LiCl) in *Suncus murinus*. It was found that both THC and CBD dose-dependently suppressed LiCl-induced vomiting; however, unlike the ability of THC to attenuate vomiting through the entire tested dose range (3-20 mg/kg), the effect of CBD was biphasic as vomiting was enhanced at higher doses (25-40 mg/kg). In a similar study, the effects of OND and THC were assessed in response to administration of the chemotherapeutic agent, cisplatin, using house musk shrews (Kwiatkowska et al. 2004). It was found that cisplatin-induced vomiting was suppressed by either compound, as well as with concomitant pretreatment of both THC and OND at doses that were ineffective alone. Importantly, this latter finding emphasizes the therapeutic potential of administering low doses of THC and OND to reduce vomiting.

Conditioned Gaping as a Measure of Nausea in Rats

Although much is known about the neurobiological basis of emesis (see Andrews & Horn, 2006), much less is known about the neural systems responsible for the sensation of nausea. Several factors contribute to this, including the subjective nature of the experience of nausea and the lack of a reliable animal model to study it

(Andrews & Horn). However, evidence suggests that conditioned taste aversion, as measured by conditioned gaping (wide triangular opening of the mouth with incisors exposed) in rats, represents a reliable animal model to investigate nausea (see Parker & Limebeer, 2008). Conditioned taste aversion learning was initially investigated by Garcia and colleagues (Garcia, Hankins, & Rusiniak, 1974), in which rats learned to associate a particular taste with toxin-induced illness. As this type of associative learning was directly related to the regulation of the internal homeostatic environment, Garcia, Hankins and Rusiniak argued that the palatability of a taste was modified by internal effects subsequent to consumption. In this way, for example, preference for a particular taste would be enhanced following an animal's recuperation from illness, whereas a taste aversion would ensue after consumption of a meal that caused illness (Garcia et al.). Interestingly, this latter type of learning was so powerful that it often resulted after a single taste-illness pairing, unlike associative learning processes pertaining to external cues (such as visual and auditory stimuli) and their external consequences (such as shock). Rats not only avoided consumption of any food that was associated with illness, but also displayed conditioned disgust (rejection) reactions when re-exposed to the illness-paired taste (Garcia et al.).

The observation that an illness-inducing drug produced conditioned disgust reactions was replicated by Grill and Norgren (1977), who developed the taste reactivity (TR) test to systematically assess responses to gustatory stimuli. Traditionally, fluid consumption tests such as one or two bottle preference tests were typically used to study flavour-illness associations. However, according to Grill and Norgren, this methodology was problematic as non-human subjects must initiate and maintain drinking behaviour

before acceptance or rejection of a taste could be accurately determined. Instead, the TR test assessed the palatability of a fluid injected directly into the oral cavity of freely moving rats, during which time the orofacial movements were video recorded for further analysis. In the event that a highly palatable solution such as sucrose was delivered intraorally, rats were observed to display ingestive reactions in the form of rhythmic mouth movements and tongue protrusions. Alternatively, bitter quinine solution elicited characteristic facial movements in the form of gaping, as well as a series of body responses (e.g. chin rubs, pawtreads) all of which were considered to be indicative of an aversion to the taste. Most interestingly, when rats were re-exposed to a sweet solution that was previously paired with a nauseating drug such as LiCl, they were also observed to display conditioned disgust reactions. Among these, gaping is the most reliable measure of aversive reactions (Breslin, Spector, & Grill, 1992). Electromyographic analysis in rats revealed a similarity between the facial musculature involved in gaping and those involved in vomiting responses among emetic species (Travers & Norgren, 1986), indicating that gaping reactions may represent an incomplete emetic response.

The TR test measures the aversiveness of a particular taste, which may not be reflected by suppressed consumption of that same taste. In fact, evidence indicates that conditioned taste aversion and conditioned taste avoidance indeed reflect different processes (Parker, 2003). Following a flavour-illness pairing, the conditioned taste comes to elicit conditioned gaping, which is likely mediated by conditioned nausea because conditioned gaping occurs only after treatment with substances capable of producing vomiting in emetic species (Parker, 1998). On the other hand, conditioned

taste avoidance occurs not only following a flavour-illness pairing but also following a pairing of a flavour with drugs of abuse such as cocaine and amphetamine that, at similar doses, are self-administered by rats (Parker, 1995). This finding indicates that nausea is not necessary for the establishment of conditioned taste avoidance. Although rats avoid a flavour paired with rewarding drugs, they do not display conditioned gaping when re-exposed to this flavour, as measured by the TR test; that is, flavours paired with rewarding drugs do not become conditionally aversive (Parker, 1995). This dissociation is not simply a difference in the sensitivity of the two measures, because Zalaquett and Parker (1989) demonstrated that even at a dose that produced weaker taste avoidance than amphetamine, LiCl produced conditioned gaping reactions while amphetamine did not. Moreover, even after 10 saccharin-amphetamine pairings, rats did not display conditioned gaping reactions when re-exposed to saccharin alone (Parker, 1991).

If conditioned gaping reactions reflect conditioned nausea-like behaviour, then attenuation of LiCl-induced nausea by anti-emetic pretreatment should interfere with the establishment of these aversive reactions. Indeed, the classic anti-emetic drugs ondansetron, and the 5-HT1A autoreceptor agonist, 8OH-DPAT, prevent the establishment of LiCl-induced conditioned gaping reactions without attenuating conditioned avoidance of that flavour during a consumption test (Limebeer & Parker, 2000; Limebeer & Parker, 2003). Therefore, because rats learn to associate a flavour with LiCl during the establishment of conditioned taste avoidance, anti-emetic drugs do not modify learning per se, but instead appear to reduce LiCl-induced nausea, whereby they selectively prevent the development of conditioned gaping reactions. As such,

conditioned gaping is a more selective measure of nausea than is suppressed consumption, in the rat.

Cannabinoid Effects on Conditioned Gaping in Rats

Cannabinoid agonists not only prevent toxin-induced emesis in species capable of vomiting, but they also prevent conditioned gaping reactions in rats. Limebeer and Parker (1999) investigated the effects of THC on toxin-induced conditioned gaping reactions in which rats were pre-treated with the cannabinoid agonist prior to receiving an intraoral infusion of 0.1 % saccharin solution and an injection of the emetic drug, cyclophosphamide. Accordingly, if THC were to reduce toxin-induced nausea, then rats would be expected to develop a weaker aversion to saccharin, as reflected by fewer disgust reactions during a subsequent drug-free test. Indeed, this is what occurred as THC attenuated the establishment of conditioned gaping reactions, as well as the expression of conditioned gaping when injected prior to the test. Subsequent studies found that LiCl-induced conditioned gaping reactions were blocked by pretreatment with the non-psychoactive cannabinoid, cannabidiol (Parker, Mechoulam and Schlievert (2002), as well as the synthetic cannabinoid, HU-210 (Parker et al. 2003). Importantly, although these cannabinoids have the ability to suppress conditioned gaping, they did not modify the strength of taste avoidance. Therefore, this effect was selective to conditioned nausea, and not attributable to an interference with learning.

Cannabinoids: Mechanism of Action

The endogenous cannabinoids, or endocannabinoids, consist of the lipid messengers *N*-arachidonoylethanolamine, or anandamide (Devane et al., 1992), and 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995), and are synthesized in an

activity-dependent manner from *N*-arachidonoyl phosphatidylethanolamine and 1, 2-diacylglycerol, respectively (Piomelli, 2003). Once released, endocannabinoids bind to metabotropic CB_1 or CB_2 receptors located on pre-synaptic axon terminals, which results in inhibition of neurotransmitter release (Piomelli), followed by carrier-mediated transport into the postsynaptic cell and enzymatic hydrolysis (Deutsch & Chin, 1993; Di Marzo et al., 1994). Anandamide is hydrolysed by the enzyme fatty-acid-amide-hydrolase (FAAH; Cravatt et al., 1996), whereas 2-AG is degraded by monoacylglycerol-lipase (MAGL; Dinh, Freund, & Piomelli, 2002), and to a lesser extent by FAAH (Di Marzo, Bisogno, Sugiura, Melck, & De Petrocellis, 1998; De Petrocellis, Cascio, & Di Marzo, 2004). As demonstrated by Lichtman and colleagues (Lichtman, Hawkins, Griffin, & Cravatt, 2002), the increases in hypomotility, hypothermia and ptosis induced by administration of a fatty-acid-amide similar to anandamide, oleamide, were enhanced in FAAH (-/-) knockout mice, whereas the behavioural effects (hypomotility, antinociception) and physiological effect of decreased rectal temperature following an injection of 2AG were equivalent among FAAH (-/-) and FAAH (+/+) mice. Therefore, the lack of behavioural enhancement among FAAH knockouts supports the role of MAGL as the principal enzyme responsible for the hydrolysis of 2-AG.

The metabolic products of endocannabinoid metabolism (see Figure A) include FAAH-mediated conversion of anandamide to the fatty-acid, arachidonic acid (AA), and ethanolamine (Cravatt et al., 1996). On the other hand, MAGL catalyzes the hydrolysis of 2AG to AA and glycerol (Karlsson, Contreras, Hellman, Tornqvist, & Holm, 1997). As FAAH and MAGL belong to the serine hydrolase superfamily (Simon

& Cravatt, 2010), it is possible to measure their activity states in proteomes using the activity-based probe, fluorophosphonate rhodamine (FP-rhodamine), followed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) separation and visualization by fluorescence scanning (Long et al., 2009). This revolutionary technique allows for a quantitative assessment of large subsets of enzymes in their native biological systems (Cravatt, Wright, & Kozarich, 2008). Although administration of exogenous AA often produces cannabinoid-like effects, it does not bind with cannabinoid receptors but rather serves as a precursor for subsequent endocannabinoid synthesis (Di Marzo et al., 1994). Importantly, AA is also a key component in the metabolic turnover to a number of eicosanoids (Smith, 1989).

Arachidonic Acid Cascade: Cannabinoids to Eicosanoids

Endocannabinoid- and phospholipid-derived AA have the potential for biotransformation into a variety of oxygenated fatty acids, known as eicosanoids (Smith, 1989), which comprise three distinct groups of signaling molecules that are formed through separate metabolic pathways. These are: 1) prostanoids 2) leukotrienes, and 3) lipid epoxides, all of which result from the hydrolysis of AA via cyclooxygenase (COX), lipoxygenase, and epoxygenase enzymes, respectively (Smith). Eicosanoids perform a broad-range of functions such as maintaining cellular homeostasis (Haeggstrom, Rinaldo-Matthis, Wheelock, & Wetterholm, 2010), regulating cardiovascular function (Imig, Simpkins, Renic, & Harder, 2011), initiating bone fracture repair (Wixted et al., 2009), as well as being implicated in certain forms of cancer (Wang & DuBois, 2010). The diversity of eicosanoid-mediated action is attributed to the multitude of pathways and receptor subtypes which mediate their

biological effects (Smith). The most well documented role for eicosanoids, however, is in mediating the inflammatory and immune response, particularly through the action of prostanoids (Davies, Bailey, & Goldenberg, 1984; Gilroy, 2010; Hata & Breyer, 2004; Larsen & Henson, 1983).

Cyclooxygenase and Prostanoids

COX-mediated hydrolysis of AA (see Figure A) occurs through two distinct enzymatic mechanisms, COX-1 and COX-2 (DuBois et al., 1998), yielding the prostanoid subfamily known as prostaglandins (PG; Poyser, 1973). Prostaglandins are a key component involved in all stages of the inflammatory response (Tiley, Coffman, & Koller, 2001); for example, PGE_2 is recruited during the onset of the inflammation, while PGD_2 levels are greatest during the final stages of the inflammatory response (Tiley, Coffman, & Koller). Selective compounds that block prostaglandin synthesis, known as non-steroidal anti-inflammatory drugs (NSAIDs), target COX enzymes specifically and are used extensively to reduce inflammation-related symptoms (Frolich, 1997). Although PGs are typically thought to contribute to the inflammatory response, recent studies have demonstrated that they also inhibit pro-inflammatory leukotrienes, as well as activate anti-inflammatory signaling molecules (Calder, 2009), indicating that PGs play an important role in both initiating and resolving inflammation. These findings suggest that drugs that manipulate anti-inflammatory pathways of prostaglandin synthesis may possess therapeutic potential, as well.

Prostanoid synthesis also occurs independent of AA, and is linked directly to the endocannabinoid system; although FAAH- and MAGL-mediated hydrolysis are the primary routes of endocannabinoid metabolism, anandamide and 2AG are also

oxygenated directly by inducible COX-2 enzymes (DuBois et al, 1998), specifically (Fowler, 2007; Woodward et al., 2008). Anandamide is a substrate for COX-2, but not COX-1, and is converted to PG ethanolamides (PGEAs) (Burstein, Rossetti, Yagen, & Zurier, 2000), while COX-2-mediated oxygenation of 2-AG yields prostaglandin glycerol esters (PG-Gs; Kozak, Rowlinson, & Marnett, 2000). These metabolic products have been shown to play a similar role as PGs in modulating immune function and pain transmission (Hu, Bradshaw, Chen, Tan, & Walker, 2008). The duration of action of PGEAs and PG-Gs is relatively short as they are rapidly converted to a number of PGs (Kozak, et al., 2001), thus, it is not surprising that their administration produces PG-like biological effects. However, Hu and colleagues (Hu, Bradshaw, Chen, Tan, & Walker) demonstrated that prostanoid receptor antagonists completely blocked the behavioural effects of PGE_2 administration, while only partially blocking those of PGE_2-G, indicating that PG-Gs exert some of their effects by a yet, undetermined, mechanism.

Endogenous Modulators of Nausea and Emesis

Although few studies have investigated the role of prostanoids in nausea in vomiting, a clear role exists for endocannabinoids in these behaviours. Much like the anti-emetic and anti-nausea properties of synthetic and plant-based cannabinoids, exogenous anandamide has been shown to suppress toxin-induced vomiting in the least shrew (Darmani, 2002), and in the ferret (Van Sickle, et al., 2001; 2005). Prolonging anandamide activity by inhibition of FAAH has been shown to interfere with cisplatin- and nicotine-induced vomiting in *Suncus murinus* (Parker et al., 2009) and the ferret (Sharkey et al., 2007). Similarly, anandamide plays an important role in conditioned nausea, as well: The FAAH-inhibitor, URB597, suppressed LiCl-induced conditioned

gaping reactions in rats (Cross-Mellor, Ossenkopp, Piomelli, & Parker, 2007), and the suppressive effects were facilitated when rats were co-treated with URB597 and exogenous anandamide. The anti-emetic and anti-nausea effects are mediated by action at CB_1 receptors, because they were reversed by pretreatment with the CB_1 antagonist/inverse agonist, SR141716 (Cross-Mellor; Parker et al., 2009). Therefore, these studies suggest that the endocannabinoid (EC) system, through the action of anandamide at CB_1 receptors, regulates nausea and vomiting. However, at present, only a few studies have evaluated the effects of 2-AG on nausea and emesis.

Role of 2-AG in the Regulation of Nausea and Emesis

Preliminary reports indicate that 2-AG may have the opposite effect as anandamide on vomiting. In the least shrew, administration of 2-AG (2.5, 5.0, or 10.0 mg/kg) has been found to dose-dependently produce vomiting responses, whereas pre-treatment with anandamide (5 mg/kg) partially blocked the emetic effects of 2-AG (Darmani, 2002). Similarly, brain 2-AG levels were increased following cisplatin-induced vomiting (Darmani et al., 2005). Contrary to the reported emetogenic effects of 2-AG, an entirely opposite set of findings were observed using a ferret model of emesis: Van Sickle et al. (2005) found that a low dose of 2-AG (0.5 mg/kg) attenuated vomiting when combined with the cannabinoid re-uptake inhibitor, VDM11, and a higher dose (2.0 mg/kg) was capable of blocking vomiting alone. Though it is unclear whether doses upwards of 2 mg/kg would produce greater anti-emetic effects in ferrets, these conflicting reports regarding the effects of 2-AG on vomiting may point to important species differences in emesis research. Nonetheless, the effect of 2-AG on vomiting in

Suncus murinus and nausea in the conditioned gaping model remains unknown, and the effect of MAGL inhibition on nausea and vomiting has also yet to be established.

As mentioned, endocannabinoid inactivation occurs rapidly through uptake and enzymatic hydrolysis (Piomelli, 2003), and this mechanism can be targeted for exploring the biological effects of endocannabinoid signaling. In the case of anandamide, pharmacological inhibitors that selectively inhibit intracellular hydrolysis by FAAH have been extensively used, leading to a better understanding of its role in the regulation of nausea and vomiting. However, until recently, an equally selective inhibitor of the 2-AG-degrading-enzyme has not existed (Long et al., 2009). Recent studies by Long et al. (2009), and Long, Nomura and Cravatt (2009) indicate that the potent and selective inhibitor, JZL184, reduces MAGL levels in mouse-brain; at doses of 4 mg/kg and 16 mg/kg, JZL184 reduced MAGL levels by 75 and 85 percent, respectively, within 0.5 h. Additionally, the lowest dose of JZL184 was found to elevate 2-AG to five times the normal level, while a high dose of 40 mg/kg resulted in a 10-fold increase in brain levels of 2-AG. Therefore, JZL184 offers a selective tool to investigate the biological effects of endogenous 2-AG, *in vivo*.

Present Study

According to previous studies, 2-AG has been found to be either anti-emetic (Van Sickle et al. 2005) or emetogenic (Darmani 2002, 2005), however the precise involvement of 2-AG in nausea, is unknown. As such, the present study was aimed at clarifying the role of 2-AG in nausea using the rat model of conditioned gaping, and vomiting using the shrew model of emesis. See Table A for a list of drugs used in the present study and their respective actions. Experiment 1 evaluated the potential of

JZL184 to modify LiCl-induced vomiting in *S. murinus*. The mechanism of action of JZL184-induced suppression of vomiting was assessed by determining if its anti-emetic effect would be reversed by the CB_1 receptor inverse agonist/antagonist, AM251. JZL184 has been shown to be less potent against rat MAGL than in humans and mice (Long, Nomura, & Cravatt, 2009) and, consistent with these results, an initial experiment confirmed that systemic administration of JZL184 (16 and 40 mg/kg, i.p.) did not modify LiCl-induced conditioned gaping reactions. Therefore, Experiment 2 evaluated the ability of exogenously administered 2-AG to modify conditioned gaping in rats. Initial experiments determined that the highest dose of 2-AG (2 mg/kg) did not produce conditioned gaping alone when explicitly paired with and infusion of 0.1 % saccharin solution. Experiment 3 evaluated the mechanism by which 2-AG suppressed LiCl-induced conditioned gaping in rats in two sub-experiments: (i) Experiment 3a assessed the potential of AM-251 (CB_1 receptor antagonist/inverse agonist) and AM630 (CB_2 receptor antagonist/inverse agonist) to reverse the effects of 2-AG on conditioned gaping. As well, the potential of AM-251 to reverse the suppressive effect of co-treatment with a high dose of JZL184 (40 mg/kg) and the highest dose of 2-AG (2 mg/kg) on conditioned gaping was assessed. It was reasoned that if the duration of action of 2-AG was prolonged by inhibition of MAGL activity, the antinausea-like effect may be produced by its agonism of CB_1 receptors. (ii) In Experiment 3b, the role of downstream metabolites of 2-AG on suppression of conditioned gaping was assessed by evaluating the potential of the COX inhibitor, indomethacin, to reverse the suppressive effect of 2-AG. Also, the potential of AA to suppress LiCl-induced conditioned gaping was evaluated. Experiment 4 assessed the alternative hypothesis that

instead of suppressing conditioned gaping by reducing nausea, 2-AG may have interfered with the sensory processing of saccharin because it was administered prior to the saccharin infusion. Therefore, in Experiment 4, rats were injected with 2-AG or vehicle immediately after intraoral exposure to saccharin solution, but 15 min prior to an injection of LiCl or saline. Finally, the potential of 2-AG to interfere with learning of an alternative classically-conditioned association, auditory fear conditioning, was assessed in rats in Experiment 5.

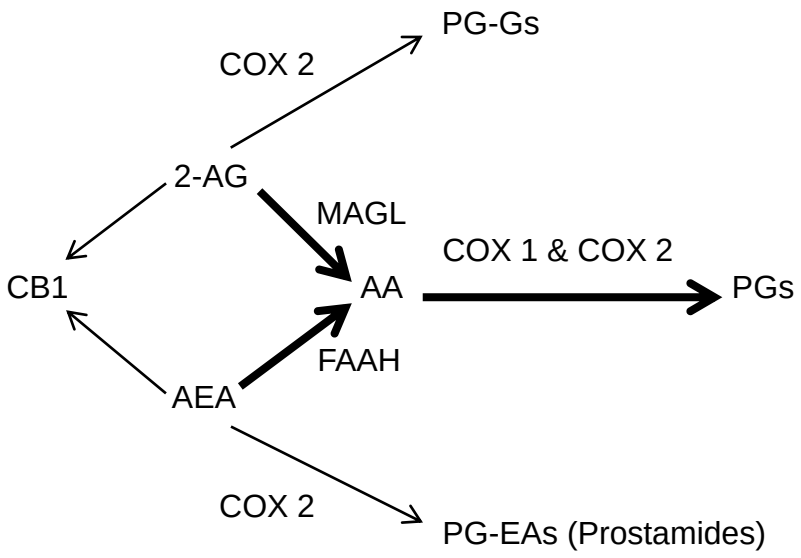
Table A

List of drugs and their respective actions

Drug	**Action**
JZL184	Selective MAGL inhibitor
2-AG	Endogenous cannabinoid; CB_1 agonist
AM-251	CB_1 antagonist / inverse agonist
AM-630	CB_2 antagonist / inverse agonist
Indomethacin	Nonselective cyclooxygenase inhibitor
Lithium Chloride	Emetogen

Figure A

Metabolic pathways of endocannabinoids



The MAGL inhibitor, JZL184, attenuates LiCl-induced vomiting in the *Suncus murinus* and 2AG attenuates LiCl-induced nausea-like behavior in rats

Running Title: 2AG and nausea/vomiting

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Background and purpose: To evaluate the role of 2AG in the regulation of nausea and vomiting using a shrew (*Suncus murinus*) model of vomiting and a rat model of nausea-like behavior, conditioned gaping. **Experimental approach:** Shrews were pretreated with JZL184, a selective MAGL inhibitor which elevates endogenous 2AG activity, one hr prior to treatment with the emetogenic drug, LiCl. The potential of exogenously administered 2AG, as well as its downstream metabolite arachadonic acid (AA), to regulate nausea-like behavior in rats was also assessed using the conditioned gaping model. The role of the CB_1 receptor, CB_2 receptor and COX inhibition in suppression of vomiting in shrews and nausea-like behavior in rats was assessed. **Key results:** JZL184 dose-dependently suppressed vomiting in shrews, an effect which was prevented by pretreatment with the CB_1 receptor inverse agonist/antagonist, AM251. *In vitro* data revealed that JZL184 inhibited MAGL activity in shrew brain tissue. In rats, 2AG suppressed LiCl-induced conditioned gaping; however, this effect was not prevented by AM251 or the CB_2 receptor antagonist, AM630. Instead, the cyclooxygenase enzyme inhibitor, indomethacin, prevented suppression of conditioned gaping by 2AG as well as AA. On the other hand, when rats were pretreated with a high dose of JZL184 (40 mg/kg), the suppression of gaping by 2AG was partially reversed by AM251. The suppression of conditioned gaping was not due to interference with learning because the same dose of 2AG did not modify the strength of conditioned freezing to a shock-paired tone. **Conclusions and implications:** The results suggest that manipulations that elevate 2AG may have anti-emetic/anti-nausea potential.

Key words: MAGL, 2AG, CB_1 receptor, cyclooxygenase, nausea, vomiting, learning, gaping, taste reactivity.

List of abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; 2AG, 2-arachidonoylglycerol; AA, arachidonic acid; AM251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl1*H*-pyrazole-3-carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3yl](4-methoxyphenyl)methanone; anandamide, *N*-arachidonoylethanolamine; CB, cannabinoid; CO_2, carbon dioxide; COX, cyclooxygenase; EC, endocannabinoid; FAAH, fatty-acid-amide hydrolase; FP, fluorophosphonate; HPβCD, 2-hydroxypropyl-β-cyclodextrin; IO, intraoral; ip, intraperitoneally; JZL184, 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; LiCl, Lithium Chloride; MAGL, monoacylglycerol-lipase; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; PG-Gs, prostaglandin glycerol esters; *S. murinus*, house musk shrew; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TR, taste reactivity; VEH, vehicle

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Introduction

One of the earliest known therapeutic effects of marihuana was that of suppression of nausea and vomiting. Considerable evidence confirms that cannabinoid agonists, such as Δ^9tetrahydrocannabinol (Δ^9-THC), WIN 212-2 and the highly potent HU-210, interfere with nausea-like behavior and vomiting in animal models (for review see Parker *et al*., 2011). These findings suggest that the endocannabinoid (EC) system is important for the regulation of nausea and vomiting (see Sanger 2007).

The endogenous cannabinoids, or endocannabinoids, consist of the lipid messengers *N*arachidonoylethanolamine, or anandamide (Devane *et al.*, 1992), and 2-arachidonoylglycerol (2AG; Mechoulam *et al.*, 1995), and are synthesized in an activity-dependent manner from *N*arachidonoyl phosphatidylethanolamine and 1, 2-diacylglycerol (Piomelli, 2003), respectively. Once released, endocannabinoids bind to metabotropic CB_1 receptors on pre-synaptic axon terminals, which results in inhibition of neurotransmitter release and are then inactivated via uptake and enzymatic hydrolysis in the postsynaptic cell (Deutsch & Chin, 1993; DiMarzo et al, 1994). Anandamide is hydrolyzed by the enzyme fatty-acid-amide-hydrolase (FAAH; Cravatt *et al.*, 1994), whereas monoacylglycerol-lipase (MAGL; Dinh *et al*, 2002) is the principal enzyme responsible for degradation of 2AG. Because FAAH and MAGL are both members of the serine hydrolase superfamily (Simon and Cravatt, 2010), their activity states in proteomes can be directly assessed by labeling with the activity-based probe fluorophosphonate rhodamine (FPrhodamine) and visualization by fluorescence scanning following SDS-PAGE separation (Long *et al*., 2009a). Recently, *in vivo*-active inhibitors of both FAAH (PF-3845) and MAGL (JZL184) have been disclosed and used

in a variety of animal models for studying heightened endocannabinoid signaling (Ahn *et al*., 2009; Long *et al*.,2009a).

Much like the anti-emetic and anti-nausea properties of synthetic or plant derived cannabinoids, exogenous administration of anandamide has been shown to suppress vomiting in the least shrew (Darmani, 2002) and in the ferret (Van Sickle *et al*., 2001; Van Sickle *et al*., 2005). Likewise prolonging the action of anandamide by inhibition of FAAH with URB597 has also been shown to interfere with cisplatin- and nicotine-induced vomiting in the *S. murinus* (house musk shrew; Parker *et al*., 2009) and in the ferret (Sharkey *et al.,* 2007). The effects are mediated by action at the CB_1 receptor because they were reversed by pretreatment with the CB_1 antagonist/inverse agonist, SR141716 (Parker *et al*., 2009). Therefore, these studies suggest that the endocannabinoid (EC) system, through the action of anandamide at CB_1 receptors, regulates vomiting.

Recent evidence indicates that the EC system not only regulates vomiting, but also regulates nausea-like behavior, as assessed by conditioned gaping reactions in rats. Although rats do not vomit, they display a selective distinctive reaction, gaping, in the taste reactivity test (Grill and Norgren, 1978) when exposed to cues (flavors or contexts) previously paired with emetic drugs (see Parker *et.al*., 2008 for review). Only drugs that produce vomiting in species capable of vomiting produce conditioned gaping reactions in rats, and conventionally employed antiemetic drugs, such as $5\text{-}HT_3$ antagonists, prevent the establishment of conditioned gaping reactions in rats. These reactions are a more selective measure of nausea than is the traditionally employed

conditioned taste avoidance measure (see Parker *et al*., 2011 for review). Cannabinoid agonists (Δ^9-THC and HU-210), like 5-HT_3 antagonists, also suppress the establishment of Lithium Chloride (LiCl)-induced conditioned gaping in rats (Limebeer & Parker, 1999; Parker *et al*., 2003). As well, the FAAH inhibitor, URB597, also prevents the establishment of LiClinduced conditioned gaping in rats in a dose-dependent manner and this effect is facilitated by co-treatment with exogenous anandamide (Cross-Mellor *et al*., 2007). These results suggest that anandamide attenuates nausea as well as vomiting.

Considerable evidence indicates a role for anandamide in the regulation of nausea and vomiting, however at present, only a few studies have evaluated the effects of 2AG on emesis and none has evaluated its effect on nausea-like behavior in rats. The endocannabinoid 2AG has been reported to be both emetic (Darmani, 2002) and anti-emetic (Van Sickle *et al*., 2005). In the least shrew, administration of 2AG (2.5, 5.0, or 10.0 mg/kg) dose-dependently produced vomiting responses, an effect that was partially blocked by pre-treatment with anandamide (Darmani, 2002). Similarly, brain 2AG levels were increased following cisplatin-induced vomiting (Darmani *et al*., 2005). On the other hand, Van Sickle *et al*., (2005) reported that in ferrets a low dose of 2AG (0.5 mg/kg) attenuated vomiting when combined with the cannabinoid re-uptake inhibitor, VDM11, and a higher dose (2.0 mg/kg) was capable of blocking vomiting alone. Although it is unclear whether doses higher than 2 mg/kg would produce greater anti-vomiting effects in ferrets, these conflicting reports regarding the effects of 2AG on vomiting may point to important species differences in the function of 2AG. The effect

of 2AG on vomiting in the *S. murinus* and nausea-like behavior in the rat conditioned gaping model remains unknown, and the effect of MAGL inhibition on nausea-like behavior or vomiting has also yet to be established.

Recently, a selective inhibitor of MAGL activity, JZL184, has been discovered (Long *et al*., 2009a; Long *et al*., 2009b), which at doses of 4 mg/kg and 16 mg/kg reduced MAGL activity in the mouse brain by 75 and 85 percent, respectively, within 0.5 h. Additionally, the lower dose of JZL184 was found to elevate 2AG to five times the normal level, while a high dose of 40 mg/kg resulted in a 10-fold increase in brain levels of 2AG. Therefore, JZL184 offers a selective tool to investigate the biological effect of endogenous 2AG, *in vivo*.

The experiments reported here evaluated the potential of JZL184 and/or exogenous 2AG to regulate vomiting in the shrew model and the nausea-like behavior in the rat model of conditioned gaping . Experiment 1 evaluated the potential of JZL184 to modify LiCl-induced vomiting in the *S. murinus*. The mechanism of action of JZL184-induced suppression of vomiting was assessed by determining if its anti-vomiting effect would be reversed by the CB_1 receptor inverse agonist/antagonist AM251. JZL184 has been shown to be less potent against rat MAGL (Long *et al*., 2009b) and, consistent with these results, an initial experiment confirmed that systemic administration of JZL184 (16 and 40 mg/kg, ip) did not modify LiCl-induced conditioned gaping reactions. Therefore, Experiment 2 evaluated the ability of exogenously administered 2AG to modify nausea-like behavior in rats using the conditioned gaping model. An initial pilot experiment determined that the highest dose

of 2AG used in these rat experiments did not produce conditioned gaping when explicitly paired with 0.1% saccharin solution. Experiment 3 evaluated the mechanism by which 2AG produced suppression of LiCl-induced conditioned gaping in rats in 2 sub-experiments: 1) Experiment 3a assessed the potential of AM251 (CB_1 antagonist/inverse agonist) and AM630 (CB_2 antagonist/inverse agonist) to reverse the effect of 2AG on gaping. As well the potential of AM251 to reverse the suppressive effect of co-treatment of a high dose of JZL184 (40 mg/kg) and 2AG on conditioned gaping was assessed. It was reasoned that if the duration of action of 2AG was prolonged by inhibition of MAGL activity, the anti-nausea-like effect may be produced by its agonism of CB_1 receptors. 2) In Experiment 3b, the role of downstream metabolites of 2AG on suppression of gaping was assessed by evaluating the potential of the Cox inhibitor, indomethacin, to reverse LiCl-induced conditioned gaping produce by 2AG. As well, the potential of AA to suppress LiCl-induced conditioned gaping was evaluated. Experiment 4 assessed the alternative hypothesis that instead of suppressing LiCl induced nausea, 2AG may have interfered with sensory processing of saccharin because it was administered prior to the saccharin infusion. Therefore, in Experiment 4, rats were injected with 2AG or VEH immediately after intraoral exposure to saccharin solution, but 15 min prior to an injection of LiCl or Saline. Finally, the potential of 2AG to interfere with learning in an auditory conditioned fear task was assessed in rats in Experiment 5.

MATERIALS AND METHODS

Animals

The subjects were male Sprague-Dawley rats weighing between 250-300 g at the start of experiments (Charles River Lab, St Constant, Quebec), as well as male (30-45 g) and female (2030 g) *S. murinus* which were bred and raised from a colony at the University of Guelph. The rats were single-housed and 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, 28% protein) and tap water except during testing, which occurred during the dark cycle. The shrews were single-housed and maintained on a diurnal light/dark cycle (7:00 am lights on; 7:00 pm lights off) with free access to food (Iams cat chow) and tap water except during testing. After all behavioral testing in each experiment, the animals were euthanized by CO_2. All experiments were approved by the Animal Care Committee of the University of Guelph and were carried out in accordance with the recommendations of the Canadian Council on Animal Care.

Drugs

All injections were delivered intraperitoneally (ip). The selective MAGL inhibitor, JZL184 (Cayman Chemicals) was prepared in a vehicle solution (VEH) of 45% 2hydroxypropyl-β-cyclodextrin (HPβCD) at concentrations of 8 mg/ml (16 mg/kg dose) or 13.33 mg/ml (40 mg/kg dose) and delivered at a volume of either 2 ml/kg (16 mg/kg dose) or 3 ml/kg (40 mg/kg dose). The doses of JZL184 were selected on the basis of Long *et al*. (2009a). The time course and doses for pretreatment were selected on the basis of previous

experiments performed by Long *et al*. (2009), demonstrating a dose-dependent increase in 2AG that, within 2 hr of administration, was sufficient to elevate brain 2AG to peak activity in mice. The drugs 2AG, AM251 (Tocris), AM630 (Tocris), Indomethacin (Sigma) and arachadonic acid (AA; Sigma) were prepared in a 1:1:18 solution of ethanol, Cremaphor, and physiological saline, respectively. For 2AG, the concentrations were 0.125 mg/ml (0.5 mg/kg dose), 0.313 mg/ml (1.25 mg/kg dose), or 0.5 mg/ml (2.0 mg/kg dose) and injected at a volume of 4 ml/kg. For AM251, the concentrations were 1.0 mg/ml (1.0 mg/kg dose), 2.5 mg/ml (2.5 mg/kg dose) or 5.0 mg/ml (5.0 mg/kg dose) administered at 1 ml/kg (1.0, 2.5 or 5.0 mg/kg). For AM630, the concentration was 2.5 mg/ml and injected at a volume of 1 ml/kg (2.5 mg/kg). For Indomethacin, the concentration was 0.83 mg/ml administered in a volume of 3 ml/kg (2.5 mg/kg). For AA, the concentration was 0.5 mg/ml administered at a volume of 4 ml/kg. The treatment drug was 0.15 M Lithium Chloride (LiCl, Sigma), which was prepared in sterile water and administered at a volume of 20 ml/kg (127 mg/kg) in rats and 60 ml/kg (390 mg/kg) in shrews. These doses were selected on the basis of their effectiveness in producing conditioned gaping (Limebeer & Parker, 2000) and vomiting (Parker *et al*., 2004), respectively.

Preparations of brain proteomes

Brains were dounce-homogenized in PBS, pH 7.5, followed by a low-speed spin (1,400 x *g*, 5 min) to remove debris. The supernatant was then subjected to centrifugation (64,000 x *g*, 45 min) to provide the cytosolic fraction in the supernatant and the membrane fraction as a pellet. The pellet was washed and resuspended in PBS

buffer by sonication. Total protein concentration in each fraction was determined using a protein assay kit (Bio-Rad). Samples were stored at –80°C until use.

FP-Rhodamine labeling of tissue proteomes.

FP-Rhodamine (Patricelli *et al*., 2001) and PF-3845 (Ahn *et al*., 2009) were synthesized as previously described. Tissue proteomes were diluted to 1 mg/ml in PBS and FP-rhodamine was added at a final concentration of 1 µM in a 50 µl total reaction volume. After 30 min at 25°C, the reactions were quenched with 4x SDS-PAGE loading buffer, boiled for 5 min at 90°C, subjected to SDS-PAGE and visualized in-gel using a flatbed fluorescence scanner (Hitachi). For experiments involving a pre-incubation with inhibitor, the reactions were prepared without FP-rhodamine. PF-3845 was added at the indicated concentration and incubated for 30 min at 37°C. FP-rhodamine was then added and the reaction was carried out exactly as described above.

Apparatus

Taste reactivity (TR) chamber: At each stage of the experiment, the rats were individually placed in a clear Plexiglas chamber (29 x 29 x 10 cm) that was resting atop a glass plate. Two 60 W lights suspended from the apparatus illuminated the chamber. To allow optimal viewing of orofacial responses, a mirror was placed at a 45 degree angle below the glass plate. Prior to being placed in the chamber, the rats were connected to an infusion pump via a section of PE 90 tubing attached to their intraoral (IO) cannula. During the course of the session, orofacial and somatic responses were recorded using a Sony video camera (Handy Cam) that was placed directly in front of the mirror and connected to a computer.

Shrew observation chamber: The shrews were observed in a similar apparatus as

the TR chamber, however, as well as being video recorded, behavioral observations were conducted live using “The Observer” (Noldus Information Technology, Sterling, VA, USA). Shrews were placed individually into the chamber and emetic episodes were observed via a mirror located at a 45 degree angle below the glass plate.

Fear conditioning chamber: the rats were conditioned in one of four chambers (30 x 24 x 40 cm; MED-Associates, Burlington, VT) constructed of aluminum (rear and side walls) and Plexiglas (door and ceiling), which were housed in sound-attenuating cabinets. The chambers were equipped with a speaker to present discrete auditory stimuli and a solid-state grid scrambler to deliver foot shocks. Background noise (65 dB) was provided by a ventilation fan that was housed in each cabinet. The activity of each rat was continuously recorded by a near-infraredbased imaging camera that was connected to a computer. Freezing behavior was assessed using “Video Freeze” (MED-Associates; Burlington, VT) software (30 fps, movement threshold: 150, min freeze duration: 90), and percent freezing behavior was calculated as the amount of time each rat was immobile per minute. The original chamber context (Context A) was used for habituation and conditioning, whereas a novel context (Context B) was created for the purpose of specifically assessing conditioned behavior to the auditory stimulus alone. Context B consisted of chambers that were equipped with an opaque white plastic insert over the floor bars and an opaque black plastic teepee overtop. The chambers were wiped down with a 1% acetic acid solution to yield a novel odour. To further distinguish from the original context, the testing room was illuminated by a dim red light (40 W), whereas the sound-attenuating cabinets were illuminated by white ambient light.

Intraoral (IO) cannulation surgery

The rats were handled three days before IO cannulation surgery. For surgery, they were anesthetized with isoflurane gas, administered the antibiotic Depocillin (0.33 mg/kg, sc; Pen Aqueous) and the nonsteroidal anti-inflammatory/analgesic drug, Carprofen (0.1 mg/kg, ip; Pfizer). A 15-gauge stainless steel needle was inserted at the mid-area on the back of the neck, and guided subcutaneously below the ear and across the cheek, where it exited into the oral cavity behind the first molar. A 10-cm section of polyethylene tubing (PE 90, I.D. 0.86mm, O.D. 1.27 mm) was inserted into the needle, which was then removed from the animal, allowing only the tubing to remain in place. Three elastomer squares (8x8 mm) were threaded onto the tubing and drawn all the way to the neck, securing the cannula firmly in place. The IO section of the cannula was held in place by a flanged-end of the tubing over a section of surgical mesh that rested flush against the skin. Twenty-four hours after surgery, rats were administered a second dose of Carprofen (0.1 mg/kg) and their health was monitored for 3 days following surgery. IO cannulae were also flushed once a day, for three days, with Chlorohexidine.

Behavioral Procedures

Experiment 1: Effect of MAGL inhibition on LiCl-induced vomiting in shrews

The shrews were each offered four meal-worms (*Tenebrio* sp.) in their home cage 15 min prior to receiving pretreatment injections. The pretreatment occurred 60 min prior to behavioral testing, in which animals received an injection of JZL184 (0, 16, 40 mg/kg) and were observed in their home cage for vomiting episodes. An additional two groups were also injected with AM251 (5 mg/kg) 5 min prior to pretreatment with

40 mg/kg JZL184 or vehicle. No shrew vomited during the 60 min period following the pretreatment. Immediately prior to the observation period, the shrews were injected with either SAL or LiCl and placed in the observation chamber for 45 min. During this time, the frequency of vomiting episodes (expulsion of fluids from stomach) and the latency (sec) to the first vomiting episode were measured. In cases when no shrew vomited, the latency measure consisted of the duration of the test session (2700 sec). The shrews were randomly assigned to the six experimental groups with approximately equal numbers of males and females in each group: VEH-LiCl (*n* = 5), JZL184 16-LiCl (*n* = 5), JZL184 40-LiCl (*n* = 5), JZL184 40-SAL (*n* = 5), AM251-JZL184 40-LiCl (*n*= 6) and AM251-VEH-LiCl (*n*=6). Group designation is denoted by the pretreatment and treatment injection, respectively.

To assess the effectiveness of JZL184 in inhibiting MAGL levels in the shrew brain, two additional groups of shrews were fed 4 meal worms 15 min prior to being injected with 40 mg/kg JZL184 (n=2; one male and one female) or VEH (n= 2; one male and one female) and placed in the observation chamber for 60 min. There was no vomiting during this 60 min observation period. Shrews were then immediately decapitated, and the brains removed within 4 min and immediately frozen in dry ice. Brains were prepared and labeled with FP-rhodamine as described in the *Materials and Methods*.

Experiment 2: Effect of exogenous 2AG pretreatment on LiCl-induced conditioned gaping in rats

The rats were handled and weighed for three consecutive days prior to IO cannulation surgery. Following surgery, they were allowed three days to recover prior

to the start of experiments, during which post-operative monitoring was conducted. All experiments consisted of adaptation, conditioning, and a TR test. The rats received a single adaptation trial to habituate them to the chamber and infusion procedure. For this session, they were placed in the TR chamber and received a 2-min IO infusion of water (reverse osmosis water infused at 1 ml/ml). On the following day, the rats received a single conditioning trial. They received a pretreatment injection of 2AG (VEH, 0.5, 1.25, or 2.0 mg/kg) 15 min prior to receiving a 2-min IO infusion of saccharin solution (0.1%) and their orofacial and somatic reactions were recorded on video. Immediately following the infusion, the rats were injected with either 20 ml/kg of LiCl (0.15M) or physiological saline (SAL) and were returned to their home cage. The 60 rats were randomly assigned to eight experimental groups, which included: VEH--LiCl (*n* = 9), VEH--SAL (*n* = 8),
0.5 mg/kg 2AG--LiCl (*n* = 7), 0.5 mg/kg 2AG--SAL (*n* = 7) 1.25 mg/kg 2AG--LiCl (*n* = 8), 1.25 mg/kg 2AG--SAL (*n* = 8), 2 mg/kg-2AG--LiCl (*n* = 7), and 2 mg/kg 2AG--SAL (*n* = 6), with group designation denoted by the pretreatment and treatment injection, respectively. Seventy-two hrs following conditioning, the rats received a drug-free TR test. During this session, they were re-exposed to a 2-min IO infusion of saccharin solution and their orofacial and somatic responses were recorded. The conditioning and testing videos were later scored for the reaction of gaping (wide opening of the mouth with bottom excisors exposed) by a rater blind to the experimental conditions using “The Observer.”

Experiment 3: Mechanism of 2AG suppression of LiCl-induced conditioned gaping in rats.

Experiment 3 evaluated potential mechanisms by which 2AG suppressed conditioned gaping in Experiment 2. In Experiment 3a, the potential of AM251 (CB_1 antagonist/inverse agonist) and AM630 (CB_2 antagonist/inverse agonist) to reverse the effect of 2AG on gaping were evaluated. As well the potential of AM251 to reverse the suppressive effect of co-treatment of a high dose of JZL184 (40 mg/kg) and 2AG on conditioned gaping was assessed. Experiment 3b determined if the cyclooxygenase (COX) enzyme inhibitor, indomethacin, would reverse the anti-nausea-like effects of 2AG or arachadonic acid (AA). In each of Experiments 3a and 3b, the rats in Groups VEH-LiCl (n=9) and 2 mg/kg 2AG –LiCl (n=7) from Experiment 2 were compared with each pretreatment group in the analyses.

Experiment 3a: Role of cannabinoid receptors in 2AG induced suppression of conditioned gaping in rats. The 70 rats included in the analysis received similar treatment as in Experiment 2, except at specified. To determine the role of the CB_1 and CB_2 receptor in the suppression of LiCl-induced conditioned gaping by 2AG, Groups AM251 (1 mg/kg)-2AG (n=8), AM251 (2.5 mg/kg)-2AG (n=9) and AM251 (5.0 mg/kg)-2AG (n=8) and AM630 (2.5 mg/kg)2AG (n=8) were injected with the antagonist 15 min prior to receiving an injection of 2AG (2 mg/kg) and 15 min later received the intraoral infusion of saccharin solution followed immediately by LiCl. Additionally, three groups were pretreated with JZL184 60 min prior to a pairing of saccharin and LiCl: Group JZL184 (n=8) was injected with JZL184 alone; Group JZL184-2AG (n=7) was also injected with 2AG 15 min prior to the conditioning trial;

Group JZL184-AM251-2AG (n=6) received the additional injection of AM251 (5.0 mg/kg) 30 min following the injection of JZL184.

Experiment 3b: Effect of the Cox inhibitor, indomethacin, on 2AG and AA suppression of LiCl-induced conditioned gaping in rats. The 48 rats included in the analysis received similar treatment as in Experiment 3a, except that they were injected with indomethacin (2.5 mg/kg) 15 min prior to pretreatment with VEH, 2AG (2 mg/kg) or AA (2 mg/kg). Fifteen min later, all rats received a pairing of saccharin infusion with LiCl. In Experiment 3b, the 48 rats were randomly assigned to the following groups: VEH-LiCl (*n* = 9), 2AG-LiCl (*n* = 7), AA-LiCl (n=9), INDOVEH-LiCl (*n* = 7), INDO-2AG-LiCl (*n* = 8), INDO-AA-LiCl (n=8).

Experiment 4: Potential of 2AG administered following saccharin to interfere with LiCl induced conditioned gaping

Experiment 4 determined if administration of 2AG following saccharin solution rather than preceding saccharin administration would interfere with LiCl-induced conditioned gaping. Experiment 4 was conducted as Experiment 2 except as specified. During conditioning, the 31 rats were intraorally infused with saccharin solution. Immediately following the infusion, they were injected with 2 mg/kg 2AG or VEH. Fifteen min later, they were injected with LiCl or saline. The groups were: Sac-VEH-Saline (n=8), Sac-2AG-Saline (n=7), Sac-VEH-LiCl (n=8), Sac-2AG-LiCl (n=8).

Experiment 5: Potential of 2AG to interfere with auditory fear conditioning

Experiment 5 evaluated whether a dose of 2AG (1.25 mg/kg) that was capable of interfering with the establishment of conditioned gaping would also interfere with

the establishment of auditory fear conditioning. Fear conditioning was based on the procedures of Maren (1999). The rats received a single 10 min habituation session, followed 24 h later by a single 7 min conditioning trial. For conditioning, the 32 rats were divided into groups (balanced for average freezing during habituation) and received an injection of either vehicle (n=16) or
1.25 mg/kg 2AG (n=16) 15 min prior to conditioning in the chamber (Context A). The rats received either 1 or 3 tone (85 dB, 2000 Hz, 10 sec) - footshock (2 sec, 0.8 mA) pairings with a 70 sec intertrial interval that began 3 min after the rats were placed into the chambers. The groups were: VEH--1 tone-shock pairing (n=8), 2AG--1 tone-shock pairing (n=8), VEH--3 tone-shock pairings (n=8), 2AG--3 tone-shock pairings (n=8). Twenty-four hours later, fear conditioning was assessed with a 10 min test that occurred in a novel context (Context B). During this test, the tone occurred after 2 min and remained on for the remainder of the 10 min session.

Data Analysis.

Behavioral data were evaluated by analysis of variance (ANOVA). In Experiment 1, the number of vomiting episodes and latency to first vomiting episode were analyzed using a single-factor ANOVA with group (VEH-LiCl, JZL16-LiCl, JZL40-LiCl, JZL40-SAL, AM-JZL40-LiCl and AM-VEH-LiCl) as a between-subjects factor. In Experiment 2, the number of gapes for each rat was entered into an ANOVA with pretreatment (at four levels: 0.0, 0.5, 1.25, and 2.0 mg/kg 2AG) and conditioning drug (at two levels: LiCl or saline) as between subjects factors. In Experiments 3a and 3b the number of gapes for each rat was entered into single-factor ANOVAs. In Experiment 4, the number of gapes were evaluated in a between factors ANOVA for

pretreatment (VEH, 2AG) and conditioning drug (Saline, LiCl). Finally in Experiment 5 separate mixed factors ANOVAs (between groups factor of pretreatment [VEH, 2AG], within groups factor of time period) were performed for freezing behavior for rats that received 1 or 3 tone-shock pairings. During conditioning, the freezing during the 70 sec preshock period was compared with each 70 sec post-shock period . During the drug-free tone test trial, each min of testing was entered into the ANOVA; the tone began during Min 3 and continued throughout the remaining 10 min test. Group differences were evaluated using planned comparison tests ($\alpha = 0.05$).

RESULTS

Experiment 1: Effect of MAGL inhibition on LiCl-induced vomiting in shrews

JZL184 dose-dependently interfered with LiCl-induced vomiting in shrews and AM251 reversed this effect. Figure 1 presents the mean (±sem) number of vomiting episodes and latency (sec) to the first vomiting episode displayed by shrews in Experiment 1. For the frequency data, statistical analyses revealed a significant main effect of group, $F(5, 26)=10.4$, $p < 0.001$; Groups JZL184 40-SAL and JZL184 40-LiCl displayed fewer vomiting episodes than all groups except JZL184 16-LiCl. Group JZL184 16-LiCl also displayed fewer vomiting episodes than the VEH-LiCl Group ($p < .05$). There was no significant difference between Groups JZL184 40-LiCl and JZL184 40-SAL. For the latency data, statistical analyses revealed a significant main effect of group, $F(5, 26)=16.8$, $p < 0.001$; Group JZL184 40-LiCl displayed a longer latency to vomit than all groups treated with LiCl except JZL184 16-LiCl. All LiCl treated groups displayed a shorter latency than Group JZL184 40-SAL. The proportion of shrews that displayed vomiting in each experimental group was as follows: JZL184 40-SAL, 0% (0/5); JZL184 40-LiCl, 60% (3/5); JZL184 16-LiCl, 100% (5/5); VEH-LiCl, 100% (5/5); AM251-JZL184 40-LiCl, 100% (6/6); AM251-VEH-LiCl, 100% (6/6).

To confirm that JZL184 inhibited MAGL in shrew tissue, brains from animals treated with vehicle or JZL184 were harvested and labeled with the serine hydrolase-directed activity probe FP-rhodamine (FP-Rh). MAGL labeled as a ~30-32 kDa doublet which is present in vehicle-treated but not in JZL184-treated shrews (Fig. 2, lanes 1-4). A second off-target of JZL184 was observed at ~60 kDa. Pretreatment of brain samples

with the FAAH inhibitor PF3845 before FP-Rh labeling blocked labeling of the upper 60 kDa band, but not the lower 60 kDa band, demonstrating that the off-target is not FAAH (Fig. 2, lanes 5-8). We suspect that this off-target is a carboxylesterase enzyme found in rodents but not humans because of its molecular weight and because JZL184 had previously been shown to have carboxylesterase off-targets in mice (Li *et al*., 2005; and Long *et al*., 2009b). Pretreatment with JZL184 before FP-Rh labeling demonstrated that MAGL was completely inhibited in vivo at 40 mg/kg (Fig. 2, lanes 9-12).

Experiment 2: Effect of exogenous 2AG on LiCl-induced conditioned gaping in rats.

Both 1.25 mg/kg and 2.0 mg/kg 2AG interfered with the establishment of conditioned gaping following a single paring of saccharin and LiCl. Figure 3 presents the mean number of gapes displayed by each group during the TR test. Statistical analyses revealed significant main effects of pretreatment drug, $F(3, 52)=9.1$, $p < 0.001$, conditioning drug, $F(1, 52)=34.6$; $p < 0.001$, as well as a pretreatment by conditioning drug interaction, $F(3, 52)=9.1$, $p < 0.001$. Rats in groups VEH-LiCl and 0.5 mg/kg-2AG LiCl displayed significantly more gaping than any other group on the test trial. No other groups differed from one another.

Experiment 3: Mechanism of 2AG suppression of LiCl-induced conditioned gaping in rats.

Neither pretreatment with the CB_1 antagonist, AM251, nor the CB_2 antagonist, AM630, reversed the suppressive effect of 2AG on LiCl-induced conditioned gaping in rats. However, when JZL184 and 2AG were co-administered, the suppressive effect of 2AG on conditioned gaping was partially reversed by AM251. Not only 2AG, but also AA, suppressed LiCl-induced conditioned gaping and these effects were reversed by the

Cox inhibitor, indomethacin.

Experiment 3a: Role of cannabinoid receptors in 2AG induced suppression of conditioned gaping in rats. Figure 4 presents the mean number of gapes displayed by each group during the TR test in Experiment 3a. Statistical analyses revealed a significant main effect of pretreatment, $F(8, 61)=10.8$; $p < 0.001$; rats pretreated with vehicle alone, JZL184 alone or JZL184-AM251-2AG displayed more gapes during the infusion of LiCl-paired saccharin solution than any other group, but did not significantly differ from one another.

Experiment 3b: Effect of COX inhibition on 2AG or AA suppression of LiCl-induced conditioned gaping in rats. In Experiment 3b, the COX inhibitor, indomethacin, reversed the suppressant effect of both 2AG and AA on LiCl-induced conditioned gaping. Figure 5 presents the mean number of gapes during the TR test of Experiment 3b. Statistical analyses revealed a significant main effect of pretreatment, $F(5, 42)=6.7$, $p < 0.001$; rats pretreated with 2AG alone or AA alone displayed fewer gapes than any other group.

Experiment 4: Potential of 2AG administered following saccharin to interfere with LiCl induced conditioned gaping

When delivered following access to saccharin solution, 2AG suppressed LiCl-induced conditioned gaping reactions, as in Experiment 2 when it was administered prior to access to saccharin solution. Figure 6 presents the mean number of gapes during the TR test of Experiment 4. The 2 by 2 ANOVA revealed significant effects of pretreatment drug, $F(1, 27) =12.6$; $p < .001$, conditioning drug, $F(1, 27) = 42.3$; $p < .001$ and a pretreatment drug by conditioning drug interaction , F

(1, 27) = 19.3; p < .001. Group Sac-VEH-LiCl displayed significantly more gaping reactions than any other group.

Experiment 5: Potential of 2AG to interfere with auditory fear conditioning in rats

Rats receiving either a single tone-shock pairing or three tone-shock pairings displayed conditioned freezing to the tone. However, pretreatment with 1.25 mg/kg 2AG did not interfere with the learning of auditory fear conditioning. Figure 7 presents the mean % conditioned freezing behavior during the pre-shock period and each post-shock period during the fear conditioning session for rats that received three pairings or one pairing of tone and shock. Among rats that received three tone-shock pairings, there was only a significant main effect of period, $F(3,42)=32.7$, $p < 0.001$, but not a significant pretreatment by period interaction, with both groups showing a similar increase in the level of post-shock freezing across parings. Among rats that received one tone-shock pairing, there was only a significant main effect of period, $F(1,14)=10.0$, $p < 0.01$; rats showed a greater % freezing during the post-shock period relative to the pre-shock period.

Conditioned freezing during the tone test is presented in Figure 8. The tone was presented at the beginning of Min 3, and the repeated measures analysis of % freezing across Min 1-3 revealed a significant effect of period for both the rats given 3 tone-shock pairings, $F(2, 28)=13.1$; $p < 0.001$, and for the rats given 1 tone-shock pairing, $F(2, 28)=9.9$; $p < 0.001$; rats displayed greater freezing during Min 3 (presentation of the tone) than during Min 1 or Min 2, indicating that they had learned the tone-shock association. There was neither a pretreatment nor a pretreatment by period interaction. Statistical analyses of % freezing during Min 3-10 for the rats that received three tone-

shock pairings also only revealed a significant main effect of period, $F(7, 98)=6.1$, $p < 0.001$, indicating that freezing decreased across minutes during the tone presentation. The pattern of results for rats that received one tone-shock pairing during Min 3-10 was similar, such that there was a significant main effect of period, $F(7, 98)= 4.5$; $p < 0.001$; with percent freezing decreasing during the tone presentation across min of testing.

DISCUSSION

Consistent with the reported anti-emetic effects of 2AG in ferrets (Van Sickle *et al.*, 2005) manipulations that elevate 2AG produced anti-vomiting/anti-nausea-like effects in the *S. murinus* and rat models respectively. The MAGL inhibitor, JZL184, which was shown to selectively inhibit MAGL activity in shrew brain tissue, attenuated vomiting produced by LiCl in the *S. murinus*. This effect appeared to be CB_1 receptor dependent, because pretreatment with AM251 reversed (or at least partially inhibited) the suppression of vomiting by 40 mg/kg JZL184. This dose of JZL184 has been shown to produce a 10 fold increase in brain levels of 2AG in mice within 0.5 hr (Long *et al.*, 2009a). Since JZL184 was relatively ineffective in rats in the conditioned gaping model of nausea-like behavior (pilot data not shown) and in other rodent models (Long *et al*., 2009a), the potential of 2AG itself to modify LiCl-induced gaping was assessed. At doses of 1.25 and 2.0 mg/kg, 2AG prevented the establishment of LiCl-induced gaping. The effect of 2AG on gaping was not reversed by the CB_1 antagonist/inverse agonist, AM251, or the CB_2 antagonist, AM630. On the other hand, the suppression of LiCl-induced conditioned gaping during co-administration of the MAGL inhibitor, JZL184, and 2AG (which presumably prolonged the duration of action of 2AG) was partially reversed by pretreatment with AM251. This latter effect suggests that under conditions

of prolonged action nausea-like behavior may have been prevented in rats by the action of 2AG on CB_1 receptors, as was vomiting in the shrews.

Downstream metabolites of 2AG also played a role in the suppression of LiCl-induced nausea-like behavior, because like 2AG, AA also suppressed conditioned gaping. Furthermore, the COX inhibitor, indomethacin, completely reversed the suppressant effects of both 2AG and AA on conditioned gaping in rats. These effects suggest that the anti-nausea-like effects of 2AG in rats are mediated by downstream metabolites of AA. Since in the presence of MAGL inhibition, the anti-nausea-like behavior produced by 2AG are CB_1 dependent, it is conceivable that co-localization of MAGL and COX may be important in the regulation of nausea and vomiting.

When released at the synapse, 2AG has a relatively short duration of action as it is rapidly degraded (e.g., Van der Steldt & Di Marzo, 2004; Fowler, 2007), which may lead to bioactive products with different physiological effects. The endocannabinoid 2AG can be hydrolyzed *in-vivo* by MAGL into AA and glycerol (Dinh *et al.*, 2002). AA is then metabolized by COX enzymes to generate prostaglandins such as PGE_2. In addition to these reactions, 2AG (and its metabolite AA) can be directly oxygenated by COX-2, producing prostaglandin glycerol esters (PG-Gs) such as PGE_2-G (Kozak *et al*., 2000; Hu et al, 2008; Woodward *et al.*, 2008). *In vitro* data suggest that PGE_2-G may act at different receptors than prostanoid receptors that mediate the effects of PGE_2, and as well, PGE_2-G is rapidly metabolized to PGE_2 (Kozak *et al.*, 2000). Recent *in vivo* data also suggests that PGE_2-G is produced by oxygenation of 2AG, which plays a role in regulation of pain and immunomodulation (Hu *et al.*, 2008). These downstream metabolites of 2AG and AA may be partially responsible for the anti-nausea-like effects

reflected in the suppression of conditioned gaping, since these effects were prevented by pretreatment with the COX inhibitor, indomethacin.

Given that 2AG was administered prior to the conditioning trial, it is also possible that the effects reported here were the result of 2AG interference with learning *per se* rather than a suppression of LiCl-induced nausea. To test the hypothesis that 2AG may be interfering with the sensory processing of the saccharin taste when administered prior to the intraoral infusion of saccharin during conditioning, in Experiment 4, rats were administered 2AG or VEH immediately following the saccharin and 15 min prior to the injection of LiCl. As was evident in Experiment 2, when 2AG was administered prior to exposure to saccharin infusion, rats displayed suppressed gaping to the LiCl-paired saccharin solution during the drug free TR test. These results confirm that the suppressed conditioned gaping was the result of a reduction in the nausea-like behavior produced by the LiCl, not of a sensory processing failure of the saccharin solution. As well, Experiment 5 evaluated the potential of 2AG to interfere with the establishment of conditioned freezing elicited by a shock-paired tone. Rats were pretreated with Vehicle or 1.25 mg/kg 2AG, a dose that was highly effective in preventing LiCl-induced conditioned gaping in Experiment 2. Then, two separate groups were given either a single tone-shock pairing or three tone-shock pairings, to ensure that ceiling effects did not mask 2AGinduced learning deficits. Rats given either one or three pairings displayed conditioned freezing during the tone test, but 2AG did not interfere with this conditioning. Therefore, it is more likely that the suppression of conditioned gaping in Experiment 2 was the result of a 2AG-mediated interference with LiCl-induced nausea-like behavior, rather than learning.

The present experiments suggest that, like anandamide (Cross-Mellor *et al* 2007; van Sickle *et al.,* 2001) and inhibitors of FAAH (Cross-Mellor *et al,* 2007; Parker *et al,* 2009; Sharkey *et al.,* 2009), 2AG and MAGL inhibitors may have therapeutic potential in the treatment of nausea and vomiting.

Figure 1 Mean (±sem) number of vomiting episodes (upper section) and mean (±sem) latency (sec; lower section) to first vomiting episode displayed by *S. murinus* during the 45 min observation period following a treatment injection of 60 ml/kg of 0.15 M LiCl or saline (Group JZL40-SAL) in Experiment 1. The various groups received different pretreatments prior to the treatment injections, including: VEH-LiCl (*n* = 5), JZL184 16LiCl (*n* = 5), JZL184 40-LiCl (*n* = 5), JZL184 40-SAL (*n* = 5), AM251-JZL184 40-LiCl (*n*= 6) and AM251-VEH-LiCl (*n*=6), with group designation denoted by the pretreatment and treatment injection, respectively. Asterisks (* $p<0.05$; ** $p< 0.01$) indicate groups which significantly differed from VEH-LiCl. Additionally, the number of shrews that vomited in each group is presented above each bar.

Figure 1

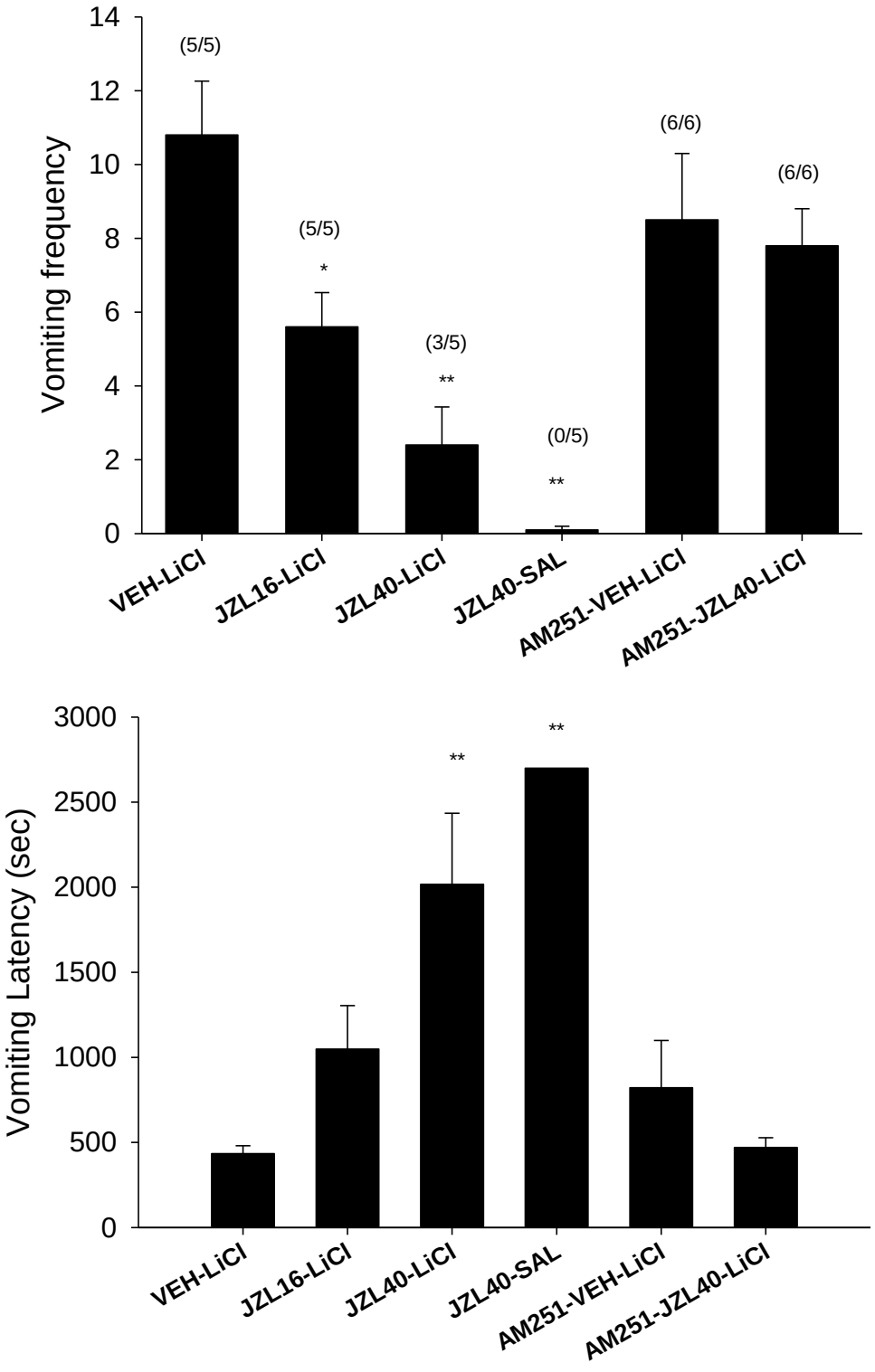
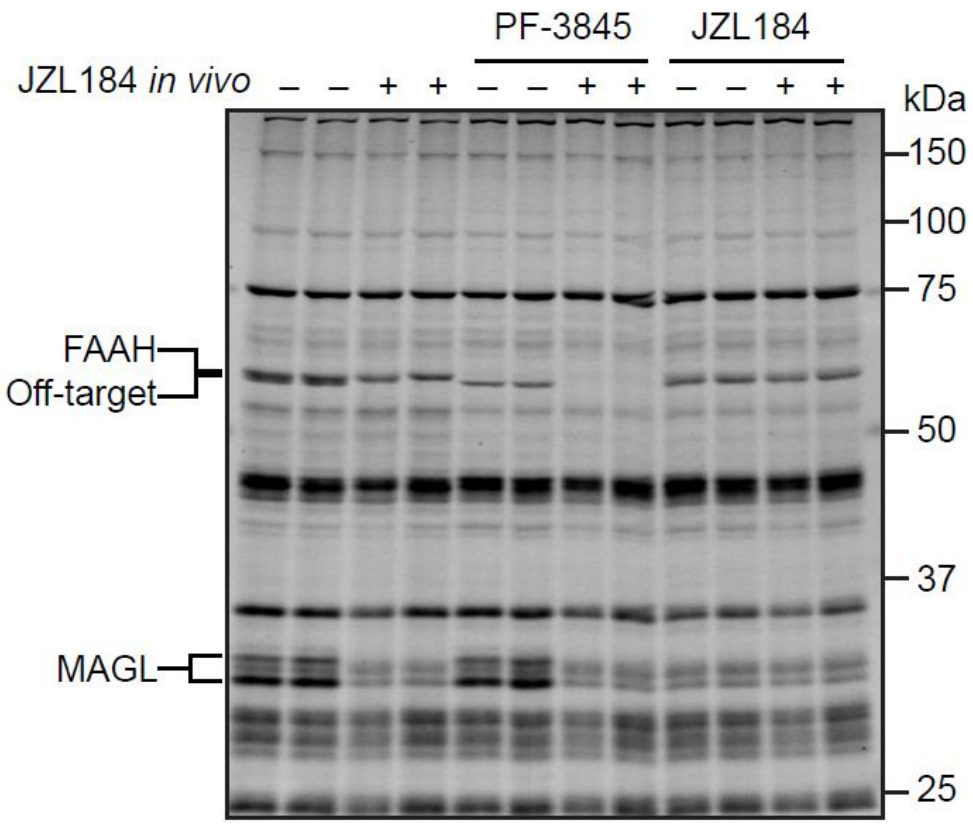
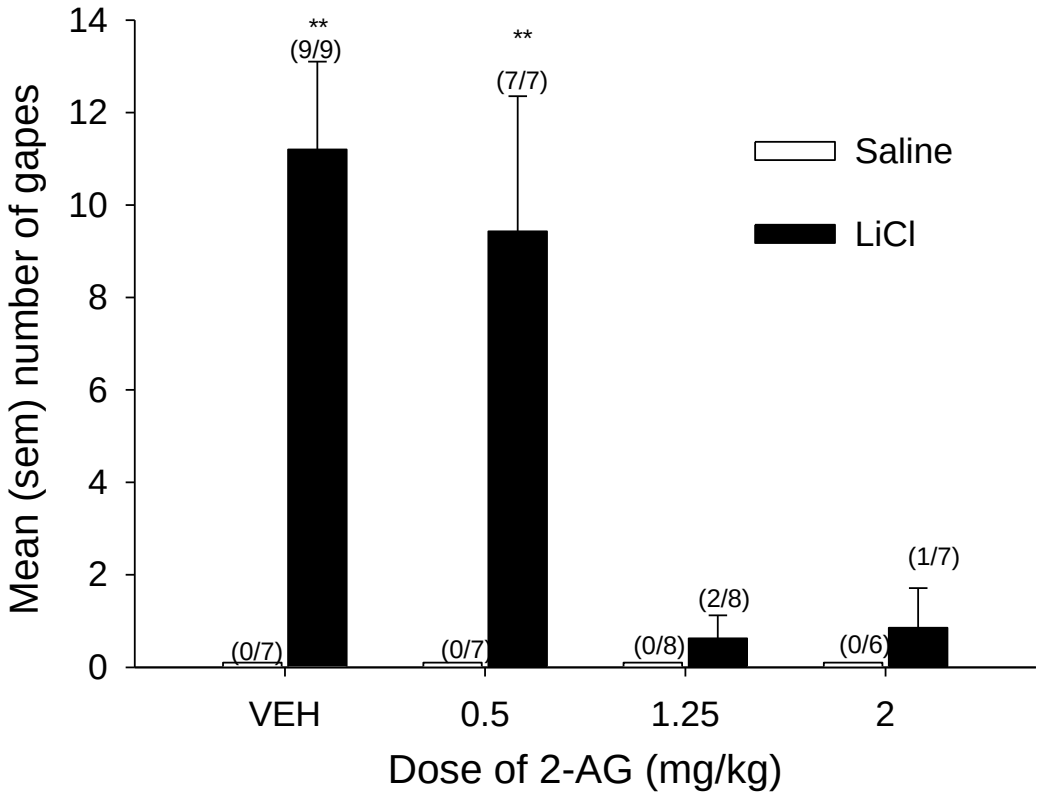

Vomiting frequency
14
12
10
8
6
4
2
0
(5/5)
(5/5)
*
(3/5)
**
(0/5)
**
(6/6)
(6/6)
VEH-LiCl
JZL16-LiCl
JZL40-LiCl
JZL40-SAL
AM251-VEH-LiCl
AM251-JZL40-LiCl
Vomiting Latency (sec)
3000
2500
2000
1500
1000
500
0
**
**
VEH-LiCl
JZL16-LiCl
JZL40-LiCl
JZL40-SAL
AM251-VEH-LiCl
AM251-JZL40-LiCl

Figure 2



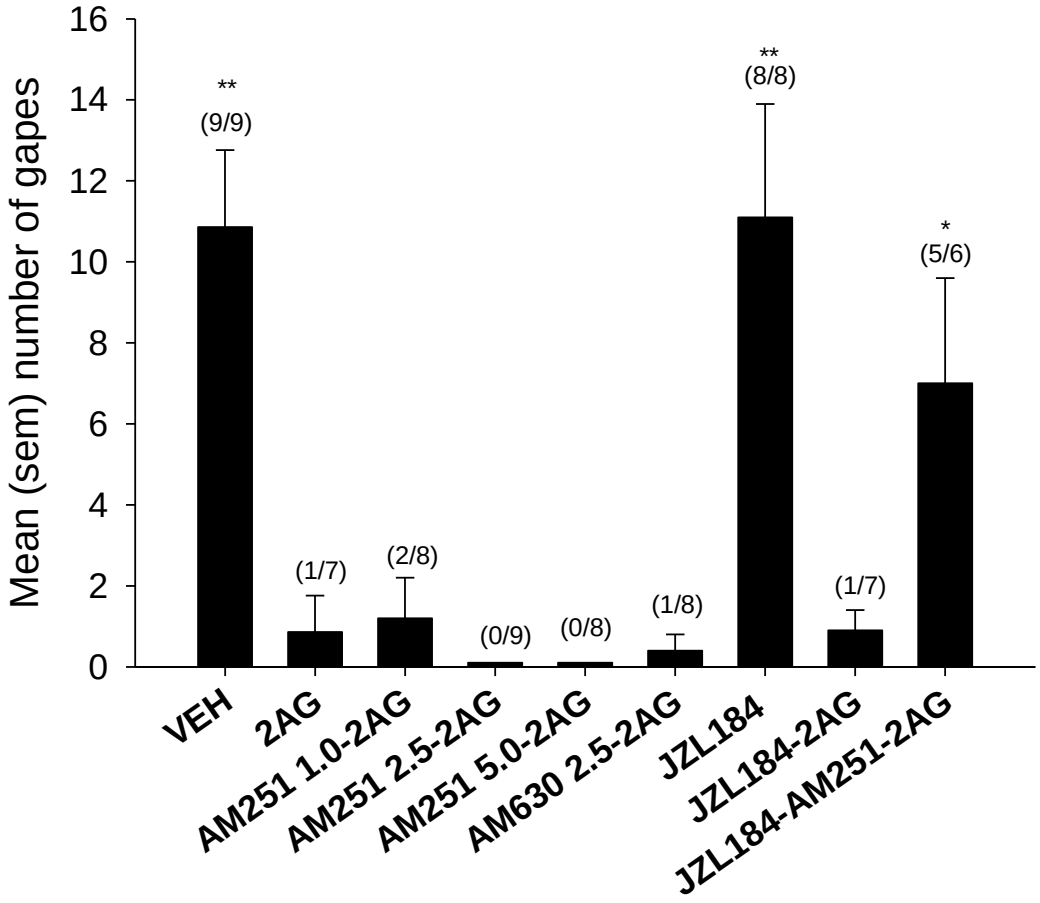
Labeling of brain membranes from shrews treated with vehicle (–) (n=2, lanes 1 and 2) or JZL184 (+) (n=2, lanes 3 and 4) using the serine hydrolase-directed activity probe FP-rhodamine (FP-Rh, 1 µM, 30 min, room temperature). Brain proteomes were also pretreated with PF-3845 (1 µM, 30 min, 37°C, lanes 5-8) or JZL184 (1 µM, 30 min, 37°C, lanes 9-12) prior to labeling by FP-Rh to confirm that the 60 kDa band and the 3032 kDa doublet are FAAH and MAGL, respectively.

Figure 3



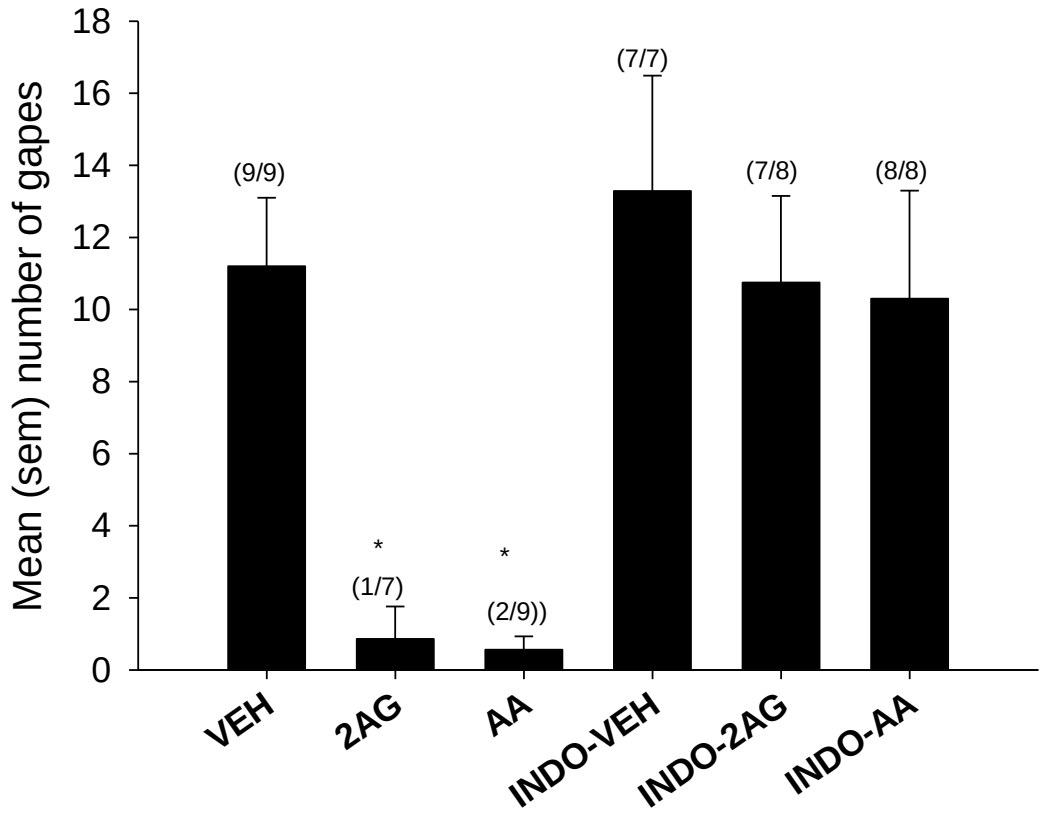
Mean (±sem) number of gapes elicited by 20 ml/kg of 0.15 M LiCl- or saline-paired 0.1% saccharin solution in rats during the drug-free TR test in Experiment 2. During conditioning, rats were pretreated with 0.0, 0.5, 1.25 or 2.0 mg/kg of 2AG. The groups were: VEH--LiCl (*n* = 9), VEH--SAL (*n* = 8), 0.5 mg/kg 2AG--LiCl (*n* = 7), 0.5 mg/kg 2AG--SAL (*n* = 7) 1.25 mg/kg 2AG--LiCl (*n* = 8), 1.25 mg/kg 2AG--SAL (*n* = 8), 2 mg/kg-2AG--LiCl (*n* = 7), and 2 mg/kg 2AG--SAL (*n* = 6), with group designation denoted by the pretreatment and treatment injection, respectively. Asterisks (** $p < 0.01$) indicate that Groups VEH-LiCl and 0.5 mg/kg 2AG-LiCl gaped significantly more than all other groups, which did not differ from one another. As well, the number of rats that gaped in each group is presented above each bar.

Figure 4



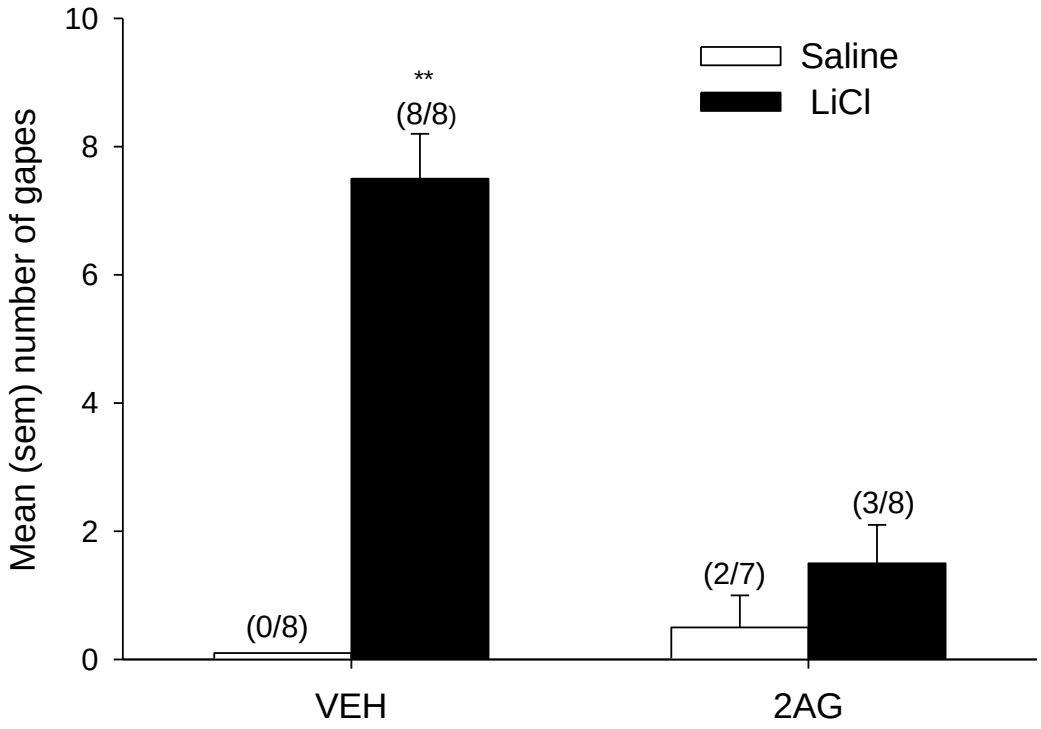
Mean (±sem) number of gapes displayed by rats during a drug free TR test in Experiment 3a. During conditioning, prior to a saccharin-LiCl pairing the rats were given the following pretreatments: VEH (n=9), 2AG (n=7), AM251 (1.0 mg/kg)-2AG (n=8), AM251 (2.5 mg/kg)-2AG (n= 9), AM251 (5.0 mg/kg)–2AG (n=8), AM630 (2.5 mg/kg)-2AG (n=8), JZL184 (n=8), JZL184-2AG (n=7) or JZL184-AM251-2AG (n=6). Asterisks (**$p < 0.01$; *$p < 0.05$) indicate that Groups VEH, JZL184 and JZL184-AM251-2AG gaped more than any other group. Additionally, the number of rats that gaped in each group is indicated above each bar.

Figure 5



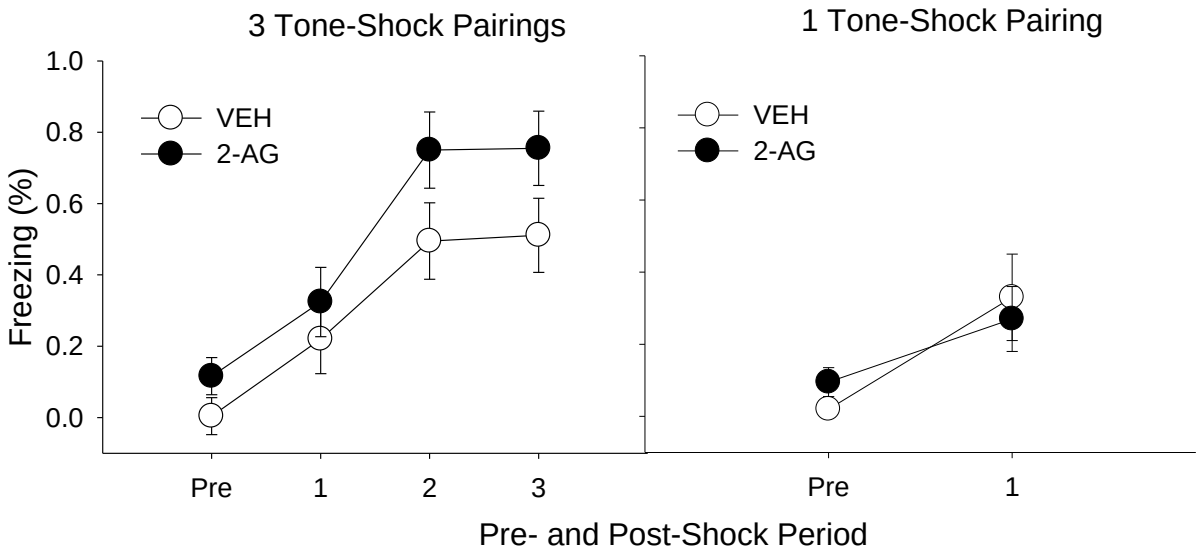
Mean (±sem) number of gapes displayed by rats in the drug free TR test in Experiment 3b. During conditioning prior to a saccharin-LiCl pairing, the rats were given the following pretreatments: VEH (n=9), 2AG (n=7), AA (n=9), INDO-VEH (n=7), INDO-2AG (n=8), INDO-AA (n=8). Asterisks (* $p < .05$) indicate that Groups 2AG and AA gaped less than any other group, with no other differences. Additionally, the number of rats that gaped in each group is indicated above each bar.

Figure 6



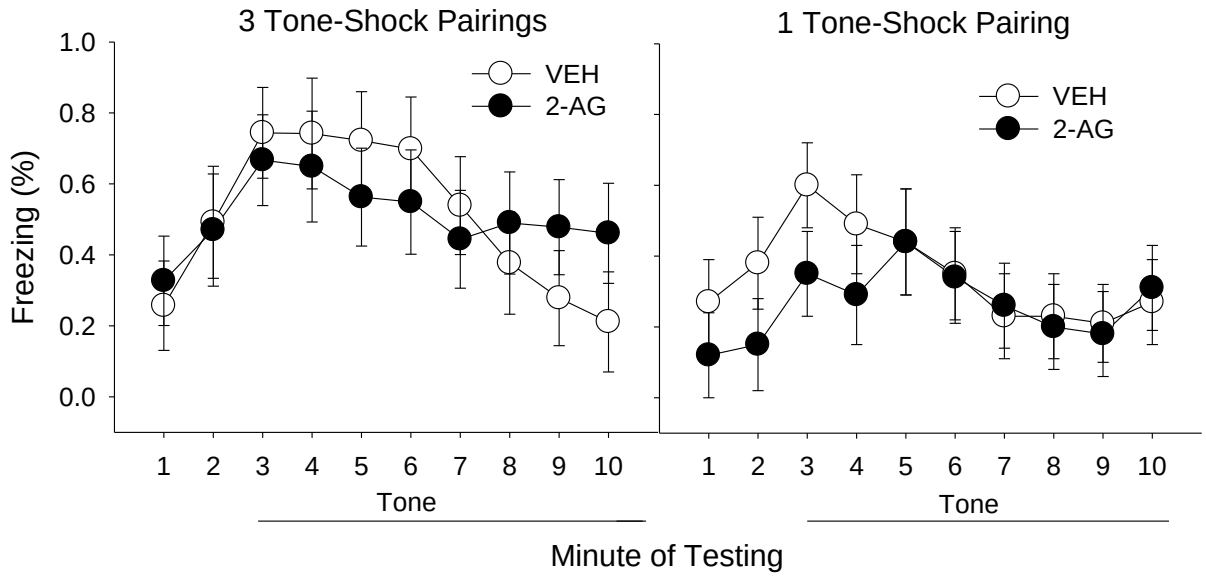
Mean (±sem) number of gapes displayed by rats during the drug free TR test in Experiment 4. Unlike Experiment 2, 2AG or VEH were injected following (rather than before) intraoral delivery of the saccharin solution and 15 min prior to injection of LiCl with 7-8 rats per group. Asterisks (** $p < 0.01$) indicate that group Sac-VEH—LiCl displayed significantly more gaping than any other group. Additionally, the number of rats that gaped in each group is indicated above each bar.

Figure 7



Mean (±sem) % freezing during the pre-shock period and each post-shock period during conditioning in Experiment 5 among rats (n=8/group) treated with either VEH or 1.25 mg/kg 2AG prior to receiving 1 or 3 tone-shock pairings.

Figure 8



Mean (±sem) % freezing during each minute of drug-free testing in Experiment 5 for rats (n=8/group) that received either VEH or 1.25 mg/kg 2AG prior to receiving either 1 or 3 tone-shock pairings during conditioning. The tone was turned on at minute 3 and remained on throughout the test.

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General Discussion

Consistent with the reported anti-emetic effects of 2-AG in ferrets (Van Sickle et al., 2005), manipulations that elevate 2-AG produced anti-vomiting and anti-nausea-like effects in the *Suncus murinus* and rat models of emesis and conditioned gaping, respectively. The MAGL inhibitor, JZL184, attenuated vomiting produced by LiCl in shrews, and this effect appeared to be mediated by CB_1 receptors, as pretreatment with the selective CB_1 receptor antagonist/inverse agonist, AM-251, reversed (or at least partially inhibited) the suppression of vomiting by 40 mg/kg JZL184. This dose of JZL184 has been shown to produce a 10-fold increase in brain levels of 2-AG in mice within 0.5 h (Long et al., 2009). However, this is the first study assessing the behavioural effects of JZL184 in the *Suncus murinus*. MAGL levels were verified in shrew brain tissue using ABPP analysis, confirming that JZL184 selectively inhibited MAGL activity in shrews. Although JZL184 was found to inhibit an off-target enzyme other than MAGL, which possesses a similar molecular weight as FAAH, it was likely a carboxylesterase enzyme found in rodents but not humans that was inhibited by JZL184 (Long, Nomura, & Cravatt, 2009). Importantly, the anti-emetic effects of JZL184 were not attributable to any off-target effects on FAAH, because *in vitro* treatment of shrew brain tissue with the FAAH inhibitor, PF3845, did not affect labeling of the carboxylesterase. Additional JZL184 treatment of shrew tissue *in vitro* did not produce greater inhibition of MAGL, indicating that a 40 mg/kg dose of JZL184 produces maximal inhibition of MAGL in shrews. These findings indicate that JZL184 is a selective tool for assessing the effects of MAGL inhibition in *Suncus murinus*.

A preliminary study in rats found JZL184 to be relatively ineffective in modulating LiCl-induced conditioned gaping, which was consistent with reports in other rodent models (Long et al., 2009). Therefore, the potential of 2-AG itself to modify LiCl-induced gaping was assessed. At doses of 1.25 mg/kg and 2.0 mg/kg, 2-AG prevented the establishment of LiCl-induced conditioned gaping. The effect of 2-AG on conditioned gaping was not reversed by AM-251, or the CB_2 receptor antagonist/inverse agonist, AM-630, indicating that the anti-nausea-like effects of exogenous 2-AG pretreatment are not mediated by CB_1 or CB_2 receptors, respectively. On the other hand, the suppression of LiCl-induced conditioned gaping reactions during co-administration of JZL184 and 2-AG, which presumably prolonged the duration of action of 2-AG, was partially reversed by pretreatment with AM-251. This latter finding suggests that, under conditions of prolonged action, nausea-like behaviour may have been prevented in rats by the action of 2-AG on CB_1 receptors, as was vomiting in shrews. Moreover, it also suggests that at higher doses JZL184 is capable of inhibiting MAGL in rats, and may be a useful tool for assessing the effects of MAGL inhibition in this animal species.

Very recent results made available after collection of the data presented in this manuscript suggest that JZL184 (8 mg/kg) produces anxiolytic-like effects in rats, which were blocked by the CB_1 antagonist, SR141716 (Sciolino, Zhou, & Hohmann, 2011). As well, intra-paw administration of JZL184 (300 μg) suppressed nociception in rats in a formalin test (Guindon, Guijarro, Piomelli, & Hohmann, 2010). Interestingly, the effects of JZL184 were enhanced by co-treatment with 2-AG and blocked by CB_1 antagonism (Guindon, Guijarro, Piomelli, & Hohmann). These recent findings suggest

that JZL184 may be useful in the study of MAGL inhibition in rats, particularly with respect to behaviours that are modulated by relatively small elevations and/or localized increases in 2-AG-mediated signaling. The use of JZL184 in such cases may, therefore, circumvent its reduced efficacy in inhibiting MAGL in rats.

Aside from the endocannabinoid-mediated effects, downstream metabolites of 2-AG also played a role in the suppression of LiCl-induced nausea-like behaviour, because, comparable to 2-AG, AA also suppressed LiCl-induced conditioned gaping. Furthermore, the COX inhibitor, indomethacin, completely reversed the suppressant effects of both 2-AG and AA on conditioned gaping in rats. This finding suggests that the anti-nausea-like effects of 2-AG in rats are mediated by downstream metabolites of AA. Because the anti-nausea-like effects of 2-AG were mediated in part by CB_1 receptors, particularly when MAGL hydrolysis of 2-AG was inhibited, it is conceivable that co-localization of MAGL and COX may be important in the regulation of nausea and vomiting.

When released at the synapse, 2-AG has a relatively short duration of action as it is rapidly degraded (Fowler, 2007; Van der Steldt & Di Marzo, 2004), and this may lead to bioactive products with different physiological effects. MAGL-mediated hydrolysis of 2-AG yields AA (Dinh, Freund, & Piomelli, 2002), which is a key component for subsequent endocannabinoid synthesis (Di Marzo et al., 1994), and also an important precursor for a number of oxygenated fatty acids (Smith, 1989). Specifically, AA is converted into a number of eicosanoids such as prostanoids, leukotrienes and lipid epoxides via COX, lipoxygenase (LOX), and epoxygenase enzymes, respectively (Smith). These lipid mediators have been implicated in a wide-range of functions, above

all in mediating the inflammatory and immune response (Davies, Bailey, & Goldenberg, 1984; Gilroy, 2010; Hata & Breyer, 2004; Larsen & Henson, 1983). Elevated prostaglandin levels are associated with all stages of the inflammatory response (Tiley, Coffman, & Koller, 2001). Thus, non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, which block prostaglandin synthesis by selectively inhibiting COX-1 and COX-2 enzymes, are an effective and widely-used pharmacological treatment for inflammation-related symptoms (Frolich, 1997). However, recent evidence indicates that prostaglandin-mediated action may also be critical in resolving inflammation, as well (Calder, 2009).

Although PGs are typically thought to contribute to the inflammatory response, recent studies have demonstrated that they also play a key role in the resolution of inflammation (Calder, 2009). For example, while increased levels of PGE_2 have been shown to mediate the onset of acute inflammation in rats (Melo Soares, Machado, Yamashiro, Melo, & Souza, 2008), *in vitro* studies have shown that it also inhibits production of monocyte-derived inflammatory cytokines in human blood cultures (Miles, Allen, & Calder, 2002). Moreover, PGE_2 also acts to inhibit LTB_4, a product of the pro-inflammatory 5-LOX pathway, and mobilize Lipoxin A_4 (Levy, Clish, Schmidt, Gronert, & Serhan, 2001) via the 15-LOX pathway (Vachier et al., 2002). This latter pathway plays an important role in resolving inflammation, because in trans-genic rabbits, overexpression of 15-LOX enzymes leads to powerful anti-inflammatory effects in response to tissue damage (Serhan et al., 2003), while Lipoxin A_4 analogs have been found to reduce pro-inflammatory gene expression in mice (Gewirtz et al., 2002). Collectively, these finding suggest that PGs belonging to the same subfamily (E_2) play

functionally opposing roles in which they both trigger and resolve the inflammatory response. Therefore, the current findings that COX-mediated products such as PGE_2 appear to mediate the anti-nausea like effects of 2-AG and AA in rats, provide further support that PGs play a supportive role in response to injury or harm.

In addition to COX-mediated conversion of AA to PGs, prostanoid synthesis also occurs by oxygenation of 2-AG directly, catalyzed by COX-2 enzymes, to produce prostaglandin glycerol esters (PG-Gs; Kozak, Rowlinson, & Marnett, 2000). These PG-Gs are rapidly converted to a number of PGs (Kozak, et al., 2001), but have also been shown to exert their effects independent of prostanoid receptors mediating the action of PGE_2 (Hu, Bradshaw, Chen, Tan, & Walker), though their precise mechanism of action remains unclear. Nonetheless, it is possible that the action of PG-Gs account for some of the anti-nausea-like effects of 2-AG in rats. However, given that AA also produced nausea-like suppressant effects comparable to that of exogenous 2-AG administration, it is likely that 2-AG-derived PG-Gs are indeed converted to a number of PGs, which in turn mediate the suppression of LiCl-induced conditioned gaping.

Given that 2-AG was administered prior to the conditioning trial, it is also possible that the effects reported here were the result of 2-AG interference with learning *per se* rather than a suppression of LiCl-induced nausea. To test the hypothesis that 2-AG may be interfering with the sensory processing of the saccharin taste when administered prior to the intra-oral infusion of saccharin during conditioning, in Experiment 4, rats were administered 2-AG or VEH immediately following the saccharin and 15 min prior to the injection of LiCl. As was evident in Experiment 2, when 2-AG was administered prior to exposure to a saccharin infusion, rats displayed

suppressed gaping to the LiCl-paired saccharin solution during the drug-free TR test. These results confirm that the suppressed conditioned gaping was the result of a reduction in the nausea-like behaviour produced by LiCl, not of a sensory processing failure of the saccharin solution.

To further evaluate the potential that 2-AG interfered with learning, Experiment 5 assessed the potential of 2-AG to interfere with the establishment of conditioned freezing elicited by a shock-paired tone. This Pavlovian conditioned association relies on a single CS-US (tone-shock) pairing analogous to that used in the model of LiCl-induced conditioned gaping (CS, saccharin; US, LiCl), and, therefore, served as an alternative behavioural paradigm to explore potential 2-AG effects on learning. Rats were pretreated with Vehicle or 1.25 mg/kg 2-AG, a dose that was highly effective in preventing LiCl-induced conditioned gaping in Experiment 2. Then, two separate groups were given either a single tone-shock pairing or three tone-shock pairings, to ensure that ceiling effects did not mask any 2-AG-induced learning deficits. It was found that rats given either one or three tone-shock pairings displayed conditioned freezing during the tone test, but 2-AG did not interfere with this conditioning. Therefore, it is more likely that the suppression of conditioned gaping in Experiment 2 was the result of a 2-AG mediated interference with LiCl-induced nausea-like behaviour, rather than learning.

Symptoms of disease or injury often include the unwanted sensations of pain and nausea. For example, characteristics associated with migraine predisposition include increased susceptibility to nausea, as well as heightened pain reflexes and hyperalgesia (Cuomo-Granston, & Drummond, 2010). This may suggest a common

pathway between pain and nausea. As interest in the role of endocannabinoids in nausea and vomiting has grown, so too has their prominence in pain research (Anand, Whiteside, Fowler, & Hohmann, 2009; Ligestri, Petrosino, & Di Marzo, 2009). Indeed, there is emerging evidence that 2-AG plays a fundamental role in mediating pain transmission. For example, dorsal horn neurons of the spinal cord express high levels of the 2-AG synthesizing enzyme, diacylglycerol lipase-alpha, which is activated in response to nociceptive input (Nyilas et al., 2009). To date, only one other study has investigated the role of 2-AG in shock-induced fear conditioning in rats: Olango and colleagues (Olango, Roche, Ford, Harhen, & Finn, 2011) examined the role of endocannabinoids in the rat dorsolateral periaqueductal grey (PAG), a key brain region mediating the descending pain pathway (Heinricher, Tavares, Leith, & Lumb, 2009), and found that freezing behaviour in response to a fear-conditioned context was associated with increased levels of 2-AG and anandamide in this brain region. Therefore, given its role in mediating pain transmission, it would not be surprising if rats treated with 2-AG were unable to acquire Pavlovian fear conditioning due to the analgesic properties of 2-AG interfering with the perception of the footshock US. However, at a dose of 1.25 mg/kg, 2-AG did not impair the acquisition of auditory fear conditioning, suggesting that the threshold for anti-nausea like properties of exogenous 2-AG administration may be lower than that required to produce analgesic effects. Although several studies have assessed the antinociceptive effects of exogenous 2-AG administration, they have been limited to localized delivery methods such as intra-plantar injections (Guindon, Desroches, & Beaulieu, 2007; Khasabova, Chandiramani, Harding-Rose, Simone, & Seybold, 2011) or intra-articular administration (Mecs et al.,

2010). Thus, the required dose of 2-AG to produce analgesic effects upon systemic administration in rats remains unclear.

Overall, the present study demonstrates that, like anandamide (Cross-Mellor, Ossenkopp, Piomelli, & Parker; Van Sickle et al., 2001; Van Sickle et al., 2005) and inhibitors of FAAH (Cross-Mellor, Ossenkopp, Piomelli, & Parker; Parker et al., 2009; Sharkey et al., 2007), 2-AG and MAGL inhibition have anti-nausea like and anti-emetic effects in the rat model of conditioned gaping and shrew model of emesis, respectively. Given that the anti-nausea like properties of 2-AG and AA appear to be mediated in part by actions of COX-derived products such as PGs, compounds that selectively target this metabolic pathway may have therapeutic potential in the treatment of nausea, specifically. Further investigation will allow a better understanding of the precise role of these metabolic products in mediating nausea.

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