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Enhancement of endocannabinoid signaling with JZL184, an inhibitor of the 2-arachidonoylglycerol hydrolyzing enzyme monoacylglycerol lipase, produces anxiolytic effects under conditions of high environmental aversiveness in rats

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Abstract

Dysregulation in signaling of the endocannabinoid 2-arachidonoylglycerol (2-AG) is implicated in hyperresponsiveness to stress. We hypothesized that blockade of monoacylglycerol lipase (MGL), the primary enzyme responsible for 2-AG deactivation *in vivo*, would produce context-dependent anxiolytic effects in rats. Environmental aversiveness was manipulated by varying illumination of an elevated plus maze. Percentage open arm time and numbers of open and closed arm entries were measured in rats receiving a single intraperitoneal (i.p.) injection of either vehicle, the MGL inhibitor JZL184 (1–8 mg/kg), the benzodiazepine diazepam (1 mg/kg), the cannabinoid CB_1 receptor antagonist rimonabant (1 mg/kg), or JZL184 (8 mg/kg) coadministered with rimonabant (1 mg/kg). JZL184 (8 mg/kg) produced anxiolytic-like effects (i.e. increased percentage open arm time and number of open arm entries) under high, but not low, levels of environmental aversiveness. Diazepam produced anxiolytic effects in either context. Rimonabant blocked the anxiolytic-like effects of JZL184, consistent with mediation by CB_1. Anxiolytic effects of JZL184 were preserved following chronic (8 mg/kg per day × 6 days) administration. Chronic and acute JZL184 treatment similarly enhanced behavioral sensitivity to an exogenous cannabinoid (WIN55,212-2; 2.5 mg/kg i.p.) 24 or 72 h following the terminal injection, suggesting a pervasive effect of MGL inhibition on the endocannabinoid system. We attribute our results to alterations in emotion rather than locomotor activity as JZL184 did not alter the number of closed arm entries in the plus maze or produce motor ataxia in the bar test. Our results demonstrate that JZL184 has beneficial, context-dependent effects on anxiety in rats, presumably via inhibition of MGL-mediated hydrolysis of 2-AG. These data warrant further testing of MGL inhibitors to elucidate the functional role of 2-AG in controlling anxiety and stress responsiveness. Our data further implicate a role for 2-AG in the regulation of emotion and validate MGL as a therapeutic target.

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Keywords

anxiety; 2-arachidonoylglycerol (2-AG); endocannabinoid; environmental aversiveness; JZL184; monoacylglycerol lipase (MGL)

1. Introduction

The endocannabinoid system is a neuromodulatory system that controls principal psychophysiological processes, including regulation of emotion and responses to stress. Dysregulation of endocannabinoid signaling is implicated in emotional disturbances, such as mood and anxiety disorders (for review see 1, 2). Blockade of cannabinoid CB_1 receptors produces anxiogenic effects (3–8), although atypical anxiolytic effects have also been reported (9, 10). Agents that prevent deactivation of the endocannabinoid anandamide exhibit psychotherapeutic efficacy in animal models (for review see 11) while minimizing psychological, physiological, and motoric side effects (12–15) associated with direct activation of cannabinoid CB_1 receptors (see also 16).

The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are deactivated by transport into cells followed by enzymatic hydrolysis (for review see 17). Anandamide is preferentially degraded by fatty-acid amide hydrolase (FAAH) (18–20), whereas 2-AG is preferentially degraded by monoacylglycerol lipase (MGL) (21–23). Selective inhibition of these hydrolytic pathways may elucidate the contributions of each endocannabinoid ligand in emotion regulation. For example, FAAH inhibitors produce anxiolytic effects in rodents (6, 15, 24–28) that are moderated by environmental aversiveness (29).

People bearing low levels of FAAH through a common single nucleotide polymorphism (C385A) in the FAAH gene exhibit diminished threat-related amygdala reactivity, a measure that negatively predicts trait anxiety (30). Yet, whether people suffering from anxiety disorders exhibit dysregulated 2-AG signaling is unknown. Individuals suffering from major depression, an affective disorder comorbid to anxiety, exhibit a selective elevation in 2-AG, but not AEA, content in serum after social stress (31). Moreover, motion (32) and orthostatic (33) stress increased plasma levels of 2-AG, but not AEA, in normal individuals after and during stressor exposure. By contrast, stress-related imagery has been reported to decrease AEA, but not 2-AG, in plasma of healthy individuals (34). These results indicate that AEA and 2-AG have distinct roles in regulating the response to stress and stress-related pathology.

Environmental stressors (i.e., maternal deprivation, cat odor) alter expression of enzymes involved in 2-AG biosynthesis and degradation in brain regions controlling emotion, such as the amygdala, hippocampus, and periaqueductal grey area of rats (35, 36). Exposure to a footshock stressor also mobilizes 2-AG in the periaqueductal gray coincident with the expression of stress-induced analgesia (37). This phenomenon is also enhanced by local inhibition of MGL, which elevates 2-AG at the same site (37). Thus, 2-AG may act in the brain to regulate physiological responses induced by aversive environmental situations. For example, social isolation is an environmental manipulation associated with symptoms of psychiatric disorders (38), whereas handling is an environmental intervention that improves many of these symptoms (39). Chronic handling attenuates both anxiety and heightened emotion induced by social isolation and concomitantly elevates 2-AG accumulation in the prefrontal cortex (40). In line with these observations, 2-AG, but not AEA, levels in the ventral striatum negatively correlate with the hyperlocomotor response elicited by the stress of exposure to a novel open field (41). The relationship between novelty-induced

locomotion and 2-AG is also abolished by subjecting rats to olfactory bulbectomy, which deafferents the ventral striatum, and produces both a hyperactive response to novelty stress and a dysregulated affective state (41). These data collectively suggest a role for 2-AG in regulating responsiveness to environmental stressors.

The recent development of MGL inhibitors (37, 42, 43) has enabled the role of 2-AG in emotion regulation to be directly investigated. A single preclinical study in mice suggests that inhibition of 2-AG degradation produces anxiolytic-like effects (44). Under baseline conditions (i.e., no stressor exposure), pharmacological inhibition of MGL with JZL184, administered acutely, produced a CB_1-dependent reduction in marble burying, a compulsive behavior exhibited in the obsessive-compulsive anxiety disorder (44). It remains unknown whether the efficacy of MGL inhibition extends across different features of emotional behavior or is dependent upon the environmental context. Moreover, whether the anxiolytic profile of a MGL inhibitor, administered acutely, persists after chronic administration has yet to be investigated. Such a demonstration is critical for validating of the therapeutic potential of MGL inhibitors for the treatment of anxiety in humans.

We tested the hypothesis that inhibition of 2-AG deactivation with the MGL inhibitor JZL184 would produce anxiolytic-like responding in the elevated plus maze in rats. We postulated that the anxiolytic efficacy of the MGL inhibitor would be dependent upon the aversiveness of the environmental context. Finally, we evaluated whether behavioral efficacy of JZL184 would be preserved following chronic administration.

2. Materials and methods

2.1. Subjects

One hundred sixty two adult male Sprague Dawley rats (270–350 g) were used. Rats from Harlan (Indianapolis, IN, USA) were used in all studies employing repeated injections of drug or vehicle. Other studies used rats from Charles River (Wilmington, MA, USA) and Harlan (Indianapolis, IN, USA). The supplier was changed from Harlan to Charles River when institutional furloughs prevented receipt of animals from Harlan. Different experimental groups were always tested together and statistical analyses were performed to verify that it was appropriate to pool animals for each experimental condition from the two suppliers. Upon arrival, rats were singly housed and kept under a 12:12 light:dark cycle (lights on at 7 AM) and temperature of 23 ± 1 °C. Rats were given *ad libitum* access to food and water and allowed at least 6 days to acclimate to the facility before testing. Cages were cleaned and refilled with sawdust bedding weekly and, therefore, this maintenance was the only handling rats received before testing. All experiments were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals and procedures were approved by the institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

2.2. Drugs

JZL184 used in single injection studies was purchased from Caymen Chemical (Ann Arbor, MI, USA). Rimonabant and JZL184 used in chronic injection studies were obtained from the National Institute on Drug Abuse Chemical Synthesis and Drug Supply Program (Bethesda, MD, USA). WIN55,212-2 and diazepam were purchased from Sigma Aldrich (St. Louis, MO, USA). All drugs were dissolved in a vehicle containing 20% DMSO and 80% emulphor: ethanol: saline in a 1:1:8 ratio. Drugs, administered either alone or in combination, were always dissolved in a volume of 1 ml/kg bodyweight to ensure that all

studies employed a uniform injection volume and handling manipulation for i.p. drug administration.

2.3 General experimental methods

2.3.1. Experiment 1: Evaluation of anxiolytic effects produced by acute (i.p.) injection of JZL184 in the elevated plus maze under varying conditions of environmental aversiveness—Rats were randomly assigned to receive a single intraperitoneal (i.p.) injection of either vehicle, the MGL inhibitor JZL184 (1, 4, or 8, or 16 mg/kg), the benzodiazepine anxiolytic diazepam (1, 2 or 3 mg/kg), or the cannabinoid CB_1 receptor antagonist rimonabant (SR141716; 1mg/kg) in the presence or absence of JZL184 (8 mg/kg). All drugs were administered 30 min before behavioral testing. After injections, rats were placed in a room adjacent to the experimental room until tested. All drugs were prepared fresh on the day of testing and dispersed in vehicle before use by vortexting. Doses of diazepam (45–47) and JZL184 (42, 48) were selected based on previous studies. Pilot studies in our laboratory in rats indicated that 8 mg/kg was the maximum behaviorally effective dose of JZL184 on elevated plus maze behavior and tests of nociception (data not shown). Limitations in drug solubility prevented testing of doses exceeding 16 mg/kg i.p. Previous research has shown that MGL blockade using JZL184 produces robust increases in 2-AG, but not AEA, in mouse brain (43). Our laboratory previously validated that, in the rat, JZL184 (local) selectively suppresses MGL but not FAAH activity *ex vivo* (49) and produces non-overlapping, modality-specific, and pharmacologically distinct antinociceptive effects from that of the FAAH inhibitor URB597 (50).

Behavioral testing was conducted during the light phase of the light:dark cycle between 11:30 AM to 6:30 PM. The elevated plus maze (EPM) was a solid black "+" shaped structure with a black matte painted floor. The apparatus was elevated 50 cm above the floor and included two open (45 × 9 cm) and two closed (45 × 9 × 38 cm) arms extending from a central platform (9 × 9 cm). Rats were placed in the central platform of the plus-shaped maze, facing an open arm opposite to the experimenter. Test sessions of 5-min duration were digitally-recorded, as previously described (51). An experimenter blind to treatment conditions quietly remained in the room during testing, hidden behind a room divider, and monitored the session. Measured behaviors were the number of open and closed arm entries and the percentage of time spent in open arms (24). Between tests, the apparatus was wiped cleaned with a chlorhexidine diacetate solution and was allowed to dry.

To induce different levels of environmental aversiveness, fluorescent lighting in the testing room was adjusted to emit low and high levels of illumination in the maze. For the low environmental aversiveness condition, illumination in the open and closed arms of the maze was 15 and 0 lux, respectively. For the high environmental aversiveness condition, illumination in the open and closed arms was 890 and 480 lux, respectively. Testing was conducted in a sound-attenuated (79 dB) environment.

2.3.2. Experiment 2: Effects of chronic and acute administration of the MGL inhibitor JZL184 on anxiety-like behavior in the elevated plus maze—Rats received repeated once daily injections of either JZL184 (8 mg/kg i.p.) or vehicle for 6 days. To control for handling effects, a third group of rats (i.e. referred to here as the acute JZL184 condition) received five once daily injections of vehicle followed by a terminal injection of JZL184 (8 mg/kg i.p.) on day 6 only. All rats were tested on the elevated plus maze under the high-light condition 30 minutes after the last injection. This study employed the same elevated plus maze testing procedure described above.

2.3.3. Experiment 3: Effects of MGL inhibition with JZL184 on locomotor activity—

Directly after elevated plus maze testing, all rats were tested for catalepsy using the bar test (52, 53). Forepaws were placed on a stainless steel bar (suspended 9 cm above a flat platform) when hindpaws remained in contact with a table, as described previously (54). Time spent immobile on the bar was measured in duplicate for each rat.

2.3.4. Experiment 4: Evaluation of behavioral sensitivity to WIN55,212-2, an exogenous cannabinoid, following chronic and acute administration of the MGL inhibitor JZL184—

Rats receiving repeated daily injections of vehicle (6 days), JZL184 (8 mg/kg i.p. × 6 days) or vehicle (5 days) followed by a terminal injection of JZL184 (8 mg/kg i.p. on day 6 only) were used to assess behavioral sensitivity to cannabinoids in the tail-flick test. Antinociceptive effects of WIN55,212-2 (2.5 mg/kg i.p.) were assessed in the same animals used in the elevated plus maze either 24 or 72 h following the terminal injection of JZL184/vehicle. This study employed methods similar to that described previously (67). Tail-flick latencies were measured three times at 2 min intervals before (baseline) and after injection of WIN55,212-2 (28–32 min post drug).

2.4. Statistics

Homogeneity of variance and group normality were validated using the Levene and Kolmogorov-Smirnov statistics, respectively. Data were analyzed by multivariate and one-way analysis of variance (ANOVA), as appropriate. Subsequent multiple comparison post hoc tests were performed, as required, to evaluate statistical significance of comparisons to control (Dunnett) and all other treatments (Fisher's Protected LSD). Planned comparison *t*-tests were used to subsequently compare effects of chronic and acute JZL184 in the elevated plus maze and determine whether effects of acute JZL184 treatment were blocked by rimonabant. Groups that received rimonabant either alone or coadministered with JZL184 did not differ and were pooled for subsequent comparisons to avoid a type II statistical error. Directionality of the tests was based upon predicted hypotheses. Comparisons that did not meet the equal variance assumption were corrected by fractional adjustment of degrees of freedom. Interclass correlation coefficients using Cronbach alpha were calculated across groups and behaviors to verify strong intra-rater reliability in behavioral coding, which ranged from $\alpha = 0.87 – 1.0$. Classic eta squared (η^2) effect size calculations were performed to gauge the amount of variance our manipulations accounted for in the dependent measures evaluated. Using Cohen's standards, eta squared values above 0.0099, 0.058, and 0.1379 can be considered small, medium, and large effects, respectively (55), although limitations of these stated criteria (e.g., overestimation of population association, dependence upon sample size) must be acknowledged (56, 57). Eta squared was calculated for ANOVAs ($SS_{\text{Factor}} / SS_{\text{Corrected Total}}$ or $SS_{\text{Factor a}} / SS_{\text{Corrected Total}} - SS_{\text{Factor a}} - SS_{\text{axb)}}$, and for t-tests ($t^2 / [t^2 + [n_1 + n_2 – 2]$). All other analyses were performed using SPSS statistical software (version 16.0; SPSS Incorporated, Chicago, IL, USA).

Antinociception in tail flick test was calculated as the percentage of maximum possible effect (% MPE), using the following formula: (Post - drug tail - flick latency - baseline) / (cut - off value - baseline) *100.

3. Results

3.1. Experiment 1

3.1.1. Under low environmental aversiveness, diazepam, but not JZL184, produces anxiolytic-like responding in the elevated plus maze—

A priori analyses revealed that diazepam-treated rats spent a greater percentage of time on the open arms compared to vehicle-treated rats under low light conditions ($t_{15} = -1.781$, $p = 0.048$; η^2

= 0.175; Fig. 1a). In addition, drug treatment increased the number of open arm entries ($F_{3,30}$ = 5.458, p = 0.004; η^2 = 0.353; Fig. 1b) without altering the number of closed arm entries (p > 0.05; Fig. 1c). Diazepam-treated rats (1 mg/kg) exhibited a greater number of open arm entries compared to vehicle- (p = 0.009; η^2 = 0.235; Fig. 1b), JZL184- (4 mg/kg) (p = 0.004; η^2 = 0.235; Fig. 1b), and JZL184 (8 mg/kg) -treated rats (p = 0.005; η^2 = 0.224; Fig. 1b). JZL184-treated rats were no different from vehicle-treated rats in the number of open arm entries (p > 0.05; Fig. 1b).

3.1.2. Under high environmental aversiveness both JZL184 and diazepam produce anxiolytic-like responding in the elevated plus maze—Under high light, drug treatment increased the percentage of time spent on the open arms of the maze ($F_{4,51}$ = 2.914, p = 0.030; η^2 = 0.186; Fig. 2a). Both JZL184 (8 mg/kg) (p = 0.039; η^2 = 0.159; Fig. 2a) and diazepam (1mg/kg) (p = 0.013; η^2 = 0.182; Fig. 2a) increased the percentage of open arm time compared to vehicle-treated rats. In addition, the drug treatments increased the number of open arm entries ($F_{4,51}$ = 2.495, p = 0.054; η^2 = 0.164; Fig. 2b) without altering the number of closed arm entries (p > 0.05; Fig. 2c). Both JZL184 (8 mg/kg) (p = 0.059; η^2 = 0.159; Fig. 2b) and diazepam (1 mg/kg) (p = 0.051; η^2 = 0.182; Fig. 2b) increased the number of open arm entries compared to vehicle-treated rats.

3.1.3. The CB_1 receptor antagonist rimonabant blocks the anxiolytic-like effects of JZL184 under high environmental aversiveness in the elevated plus maze—*A priori* analyses revealed that JZL184 (8 mg/kg)-treated rats spent a greater percentage of time on the open arms (t_{26} = −2.525, p = 0.009; η^2 = 0.197; Fig. 3a) and exhibited more open arm entries (t_{26} = −2.186, p = 0.019; η^2 = 0.155; Fig. 3b), relative to vehicle-treated rats. Rimonabant (1 mg/kg) also blocked the JZL184-induced increase in both open arm time and open arm entries; rats receiving JZL184 (8 mg/kg) exhibited a greater percentage of time spent on the open arms (t_{32} = 1.813, p = 0.040; η^2 = 0.096; Fig. 3a) and a greater number of open arm entries (t_{32} = 2.955, p = 0.003; η^2 = 0.220; Fig. 3b) compared to rats treated with rimonabant (1 mg/kg) in either the presence or absence of JZL184 (8 mg/kg). Percentages of open arm time and numbers of open arm entries did not differ between rats receiving vehicle or rimonabant (1 mg/kg) (p > 0.05; Fig. 3a–b). Moreover, percentages of open arm time and the numbers of open arm entries did not differ between rats receiving rimonabant in the absence or presence of JZL184 (8 mg/kg) (p > 0.05; Fig. 3a–b). No drug altered the number of closed arm entries (p > 0.05; Fig. 3c).

3.1.4. JZL184 produces context-dependent anxiolytic-like effects in the elevated plus maze whereas high environmental aversiveness produces anxiogenic-like effects—Drug treatment altered both the percentage of open arm time ($F_{2,58}$ = 5.376, p = 0.007; η^2 = 0.162; Fig. 4a) and frequency of open arm entries ($F_{2,58}$ = 7.684, p = 0.001; η^2 = 0.176; Fig. 4b). Both JZL184 (8 mg/kg) (p < 0.05; η^2 = 0.162; Fig. 4a) and diazepam (1 mg/kg) (p < 0.05; η^2 = 0.085; Fig. 4a) increased the percentage open arm time relative to vehicle. Diazepam (1 mg/kg) also increased the number of open arm entries relative to vehicle (p < 0.05; η^2 = 0.095; Fig. 4b). However, JZL184 (8 mg/kg)-treated rats exhibited a lower percentage of open arm time (p < 0.05; η^2 = 0.085; Fig. 4a) and fewer open arm entries (p < 0.05; η^2 = 0.095; Fig. 4b) relative to diazepam (1 mg/kg). Rats tested under high light exhibited fewer open arm entries ($F_{1,58}$ = 3.814, p = 0.056; η^2 = 0.046; Fig. 4b) relative to rats tested under low light, but did not reliably differ in the percentage of time spent on the open arms of the elevated plus maze (p > 0.05; Fig. 4a). Drug treatment differentially altered the frequency of open arm entries depending on lighting condition during testing ($F_{2,58}$ = 3.859, p = 0.027; η^2 = 0.092; Fig. 4b). When tested under low light, diazepam (1mg/kg) elicited a greater number of open arm entries relative to rats treated with the same dose of diazepam (1 mg/kg) under high light (p < 0.05;

η^2 = 0.219; Fig. 4b), vehicle under either low ($p < 0.05$; η^2 = 0.232; Fig. 4b) or high ($p < 0.05$; η^2 = 0.179; Fig. 4b) light, or JZL184 (8 mg/kg) under either low ($p < 0.05$; η^2 = 0.232; Fig. 4b) or high ($p < 0.05$; η^2 = 0.179; Fig. 4b) light. When tested under high light, rats treated with either JZL184 (8 mg/kg) ($p < 0.05$; η^2 = 0.140; Fig. 4b) or diazepam (1 mg/kg) ($p < 0.05$; η^2 = 0.164; Fig. 4b) exhibited a greater number of open arm entries relative to vehicle. No drug or lighting treatment altered the frequency of closed arm entries ($p > 0.05$; Fig. 4c).

3.2. Experiment 2: Chronic and acute administration of JZL184 produces comparable anxiolytic-like responding in the elevated plus maze under high environmental aversiveness

A priori analyses revealed that JZL184, administrated either chronically (8 mg/kg i.p. per day for 6 days) or acutely (once daily injections of vehicle for 5 days followed by a terminal injection of 8 mg/kg i.p. on day 6) elicited greater percentage of open arm time (t_{33} = 2.063, p = 0.024; η^2 = 0. 114; Fig. 5a) and number of open arm entries (t_{27} = 3.031, p = 0.003; η^2 = 0.254; Fig. 5b) than vehicle-treated rats. Neither treatment altered the number of closed arm entries ($p > 0.05$; Fig. 5c). Percentages of open arm time and the number of open arm entries did not differ between rats receiving chronic or acute treatment with JZL184 (8 mg/kg) ($p > 0.05$; Fig. 5a–b).

3.3. Experiment 3: Diazepam but not JZL184 produces catalepsy

Acute drug treatments differentially altered ($F_{6,62}$ = 6.119, p = 0.000; η^2 = 0.372; Fig. 5a) time spent immobile in the bar test. The high dose of diazepam (3 mg/kg) increased immobility time relative to either vehicle, the low dose of diazepam (1 mg/kg), or a range of doses (1, 4, 8, 16 mg/kg) of JZL184 (p = 0.000; Fig. 6a). JZL184 did not alter descent latencies in the bar test relative to vehicle-treated rats at any dose ($p > 0.05$; Fig. 6a). Immobility times were similar in groups receiving either the middle (2 mg/kg i.p.) or high (3 mg/kg i.p.) doses of diazepam ($p > 0.05$; Fig. 6a). Neither chronic (8mg/kg i.p. × 6 days) nor acute (8mg/kg i.p. on day 6 only) administration of JZL184 altered immobility times in the bar test relative to vehicle-treated rats ($F_{2, 19}$ = 2.796, p = 0.088; Fig. 6b). Immobility times did not differ between groups (p >0.05, Fig. 6b).

3.4. Experiment 4: Chronic and acute administration of JZL184 enhance behavioral sensitivity to WIN 55, 212-2, an exogenous cannabinoid

Behavioral sensitivity to WIN55,212-2, a synthetic cannabinoid, was evaluated in the tail-flick test 24 or 72 h after the terminal JZL184/vehicle injection in the same rats used in the elevated plus maze test (see Fig. 6). The maximal possible antinociceptive effect of WIN55, 212-2 (2.5 mg/kg i.p.) was increased ($F_{2,30}$ = 5.180, p = 0.012; η^2 = 0.257; Fig. 7) in rats treated either chronically (8 mg/kg i.p. × 6 days; p = 0.014; η^2 = 0.235; Fig. 7) or acutely (8 mg/kg i.p. on day 6; p = 0.006; η^2 = 0.282; Fig. 7) with JZL184, relative to vehicle treatment. WIN55, 212-2-induced antinociception did not differ between chronic and acute JZL184-treated groups at either timepoint.

4. Discussion

The present studies identified context-dependent anxiolytic effects of JZL184, an inhibitor of the 2-AG hydrolyzing enzyme MGL, that were dependent upon the level of environmental aversiveness. Under baseline conditions (low environmental aversiveness), preventing deactivation of 2-AG with JZL184 did not alter behavioral responding in a well characterized test of anxiety, the elevated plus maze. However, under more stressful circumstances (high environmental aversiveness), JZL184 reduced anxiety-like behavior in the elevated plus maze in a CB_1-dependent manner. Furthermore, the anxiolytic effects of

JZL184 were preserved following chronic administration, suggesting that they did not undergo tolerance in our study. JZL184 produced a distinct anxiolytic profile from standard treatment with the benzodiazepine diazepam. The anxiolytic ability of JZL184 is comparable to diazepam in the magnitude of effectiveness, but, unlike diazepam, emerges only under more stressful circumstances. We attribute the effects of JZL184 to alterations in emotion, and not to locomotor activity, as JZL184, administered chronically or acutely, did not produce motor impairment in the elevated plus maze or catalepsy in the bar test. Our results demonstrate that JZL184 has beneficial and environmentally-dependent effects on anxiety in rats, presumably via inhibition of MGL-mediated hydrolysis of 2-AG.

A striking observation of our study was that animals treated chronically with JZL184 (8 mg/kg per day for 6 days) showed no loss in anxiolytic efficacy in the elevated plus maze. Chronic (8 mg/kg i.p. × 6 days) and acute (8 mg/kg i.p. on day 6) treatment with JZL184 produced equally efficacious anxiolytic profiles (i.e. similar magnitude increases in percentage open arm time and number of open arm entries) and also produced similar levels of behavioral sensitization to the antinociceptive effects of WIN55,212-2 (2.5 mg/kg i.p.). Our results suggest that JZL184, administrated either chronically or acutely in rats, produces a prolonged blockade of endocannabinoid deactivation without producing tolerance, at least with the current dosing schedule. By contrast, tolerance and receptor desensitization are observed in mice following repeated administration of higher doses of JZL184 (40 mg/kg i.p. for 6 days) (16), a treatment regimen that produces approximately 85% inhibition of MGL activity after a single injection (42, 43). Differences in species selectivity (rats vs. mice) and dose (8 mg/kg per day for 6 days vs. 40 mg/kg per day for 6 days) of JZl184 employed may account for differences between the present study and that by Schlosburg et al. (2010). Our results suggest that a sustained elevation of endocannabinoid tone was still present 72 h following acute or chronic injection of JZL184 in the rat. However, stimulation-contingent 2-AG mobilization under conditions of high environmental aversiveness was required to unmask anxiolytic effects of JZL184 in the elevated plus maze. Nonetheless, the fact that the MGL inhibitor JZL184 remained effective in suppressing anxiety-like behavior after repeated administration implies that MGL inhibitors may show therapeutic potential as anxiolytics in humans.

Our findings are congruent with other data which suggest that benzodiazepines such as chlordiazepoxide produce anxiolytic-like responding in the elevated plus maze regardless of the degree of environmental aversiveness present during testing (29). Previous studies substantiate that increasing illumination during the elevated plus maze test is aversive (29, 58) and that effects of the endocannabinoid system or endocannabinoid modulators on anxiety are uncovered by altering environmental aversiveness (25, 29, 58). Similar to inhibition of 2-AG hydrolysis with JZL184 (present report), inhibition of anandamide hydrolysis with URB597 produced anxiolytic-like effects in the elevated plus maze only when tested under high light (29). Likely brain regions for which JZL184 exerts its effects on anxiety-like behavior in our study may be analogous to those implicated in studies employing site-specific modulation of anandamide hydrolysis. For example, anxiety-like responding was reduced by local inhibition of FAAH in the basolateral amygdala complex (59), dorsolateral periaqueductal grey area (60), hippocampus (61), infralimbic region (62), and prefrontal cortex (63). Future microinjection studies are needed to determine whether these regions mediate the effects of systemic JZL184 on anxiety described here. Together, these data suggest that both anandamide and 2-AG regulate the behavioral expression of emotion, especially under high stress circumstances.

Evidence supports both overlapping and separate roles for 2-AG and anandamide signaling in the brain to regulate stress. For example, both 2-AG and anandamide are recruited to mimic and occlude glucocorticoid-induced suppression of glutamate release in the

hypothalamus (64) and attenuate anxiety-related behaviors induced by repeated drug exposure (65). Similarly, acute inhibition of either 2-AG or anandamide hydrolysis attenuated both defensive burying (44) and rimonabant-precipitated somatic withdrawal in Δ^9-tetrahydrocannabinol-dependent mice (66). However, several lines of evidence support a unique role for each endocannabinoid ligand in brain circuitry regulating emotion after footshock exposure (i.e., during stress-induced analgesia) (37), acute corticosterone treatment (67), chronic restraint stress (68–71), chronic unpredictable stress (72), and maternal deprivation (73). Selective targeting of these chemical pathways using tools that prevent the synthesis or hydrolysis of endocannabinoids are needed to extend these findings and determine the relative contribution and potential synergism of endocannabinoids in emotion regulation.

Our results, along with those of others (6, 13, 15, 24, 28, 60), suggest that inhibition of endocannabinoid-degrading enzymes prolongs endocannabinoid signaling to produce monophasic, treatment-like effects on anxiety. In our study, similar levels of behavioral sensitization to exogenous cannabinoids were observed in animals receiving either chronic or acute treatment with JZL184. This observation further underscores the ability of JZL184 to enhance endocannabinoid tone. These data stand in contrast to those generated using exogenous cannabinoid CB_1 receptor agonists or antagonists in preclinical models, wherein biphasic effects or both anxiogenic and anxiolytic effects are dose-dependently produced (for review see 74, 75). Similarly, clinical studies employing cannabinoid CB_1 receptor antagonist/inverse agonists suggest that blocking the function of endocannabinoids may produce anxiety, depression, and suicidal ideation (76–78). However, these effects may be due to excessive cannabinoid CB_1 receptor blockade and limited to patients at high risk for mental illness (75). Pro-cannabinoid agents, such as MGL inhibitors, that enhance endocannabinoid tone without acting directly on CB_1 receptors, may exhibit better clinical utility relative to CB_1 receptor blockers. Indirect methods that elevate endocannabinoid tone may offer a more circumscribed and beneficial spectrum of physiological effects compared to direct activation of CB_1 receptors. MGL inhibitors may, consequently, offer lasting efficacy in reducing anxiety with minimal tolerance or untoward psychogenic side effects (i.e. compared to either direct cannabinoid agonists or benzodiazepines). More work is necessary to determine whether pharmacological inhibitors of MGL may be used to exploit the therapeutic potential of the endocannabinoid system to suppress anxiety in humans.

5. Conclusions

Here we demonstrate that JZL184 has beneficial and environmentally-dependent effects on anxiety in rats, presumably via inhibition of MGL-mediated hydrolysis of 2-AG. Anxiolytic efficacy of JZL184 was revealed under conditions of high environmental aversiveness. Moreover, the anxiolytic profile of JZL184 showed no evidence of undergoing tolerance following repeated administration of the drug. These data warrant further testing of inhibitors of 2-AG deactivation in animal models of anxiety to elucidate the role of 2-AG in emotion regulation. Our data implicate a pivotal role for 2-AG in controlling anxiety and stress responsiveness and further validate MGL as a therapeutic target.

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Abbreviations

ANOVA Analysis of variance

AEA	anandamide
2-AG	2-arachidonoylglycerol
CB_1	cannabinoid receptor type 1
C385A	common single nucleotide polymorphism
EPM	elevated plus maze
FAAH	fatty acid amide hydrolase
i.p	intraperitoneal
MAFP	14-eicosatetraenyl-methyl ester phosphonofluoridic acid
MGL	monoacylglycerol lipase
SS	and sums of squares

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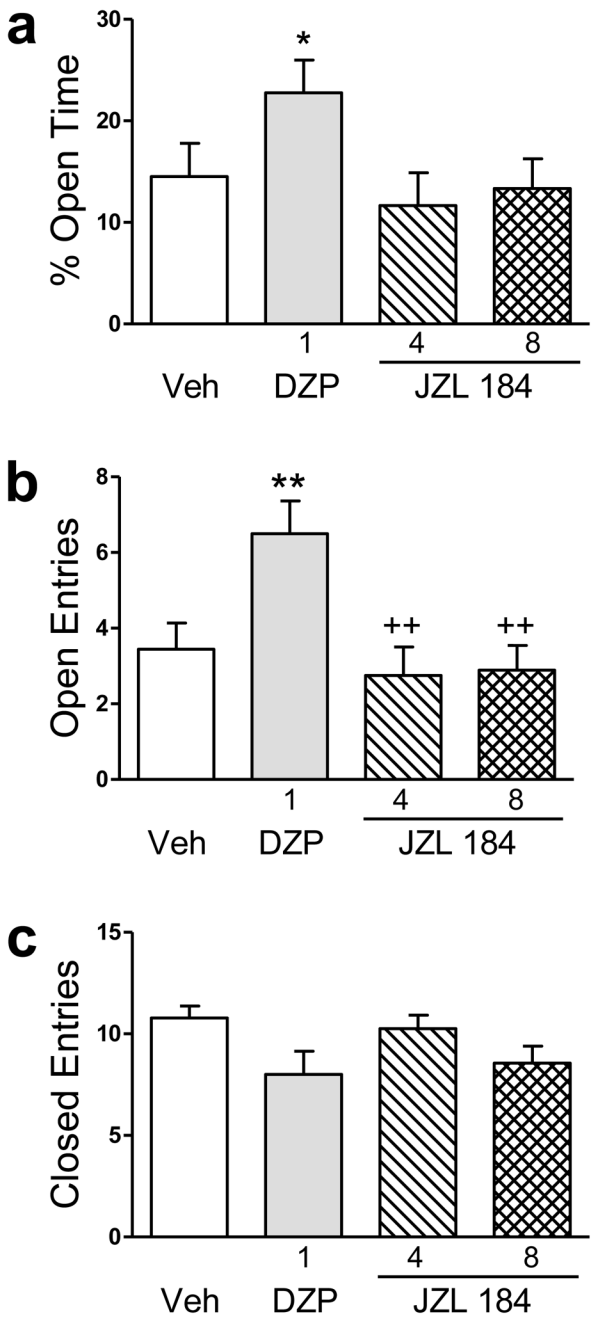

Figure 1. Under conditions of low environmental aversiveness, diazepam, but not JZL184, produced anxiolytic-like responding in the elevated plus maze

Under low light, diazepam (DZP, 1 mg/kg), but not JZL184 (4, 8 mg/kg), increased both the (a) percentage of open arm time and (b) frequency of open arm entries, relative to vehicle. Under low light, neither JZL184 (4, 8 mg/kg) nor diazepam (DZP, 1 mg/kg) altered (c) the frequency of closed arm entries, relative to vehicle. Data are mean ± S.E.M (n = 8 – 9). $^{**}p < 0.01$ vs. vehicle (ANOVA, Dunnett post hoc, one tailed); $^{*}p < 0.01$ vs. Vehicle (t-test, one tailed); $^{++}p < 0.01$ vs. diazepam (ANOVA, Dunnett post hoc, two tailed).

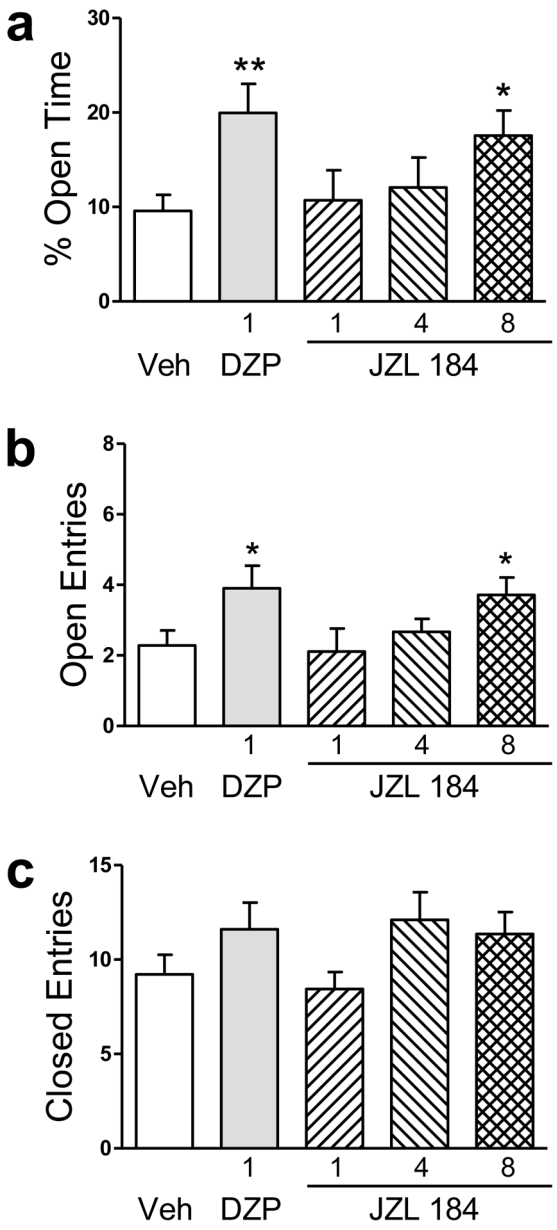

Figure 2. Under conditions of high environmental aversiveness, both JZL184 and diazepam produced anxiolytic-like responding in the elevated plus maze

Under high light, JZL184 (8 mg/kg) dose dependently increased both the (a) percentage of open arm time and (b) frequency of open arm entries, relative to vehicle. Diazepam (DZP, 1 mg/kg) mirrored (a–b) the changes induced by JZL184 (8 mg/kg). Under high light, neither JZL184 (4, 8 mg/kg) nor diazepam (DZP, 1 mg/kg) altered (c) the frequency of closed arm entries, relative to vehicle. Data are mean ± S.E.M (n = 9 – 14). $^{**}p < 0.01$, $^{*}p < 0.05$ vs. vehicle (ANOVA, Dunnett post hoc, one tailed).

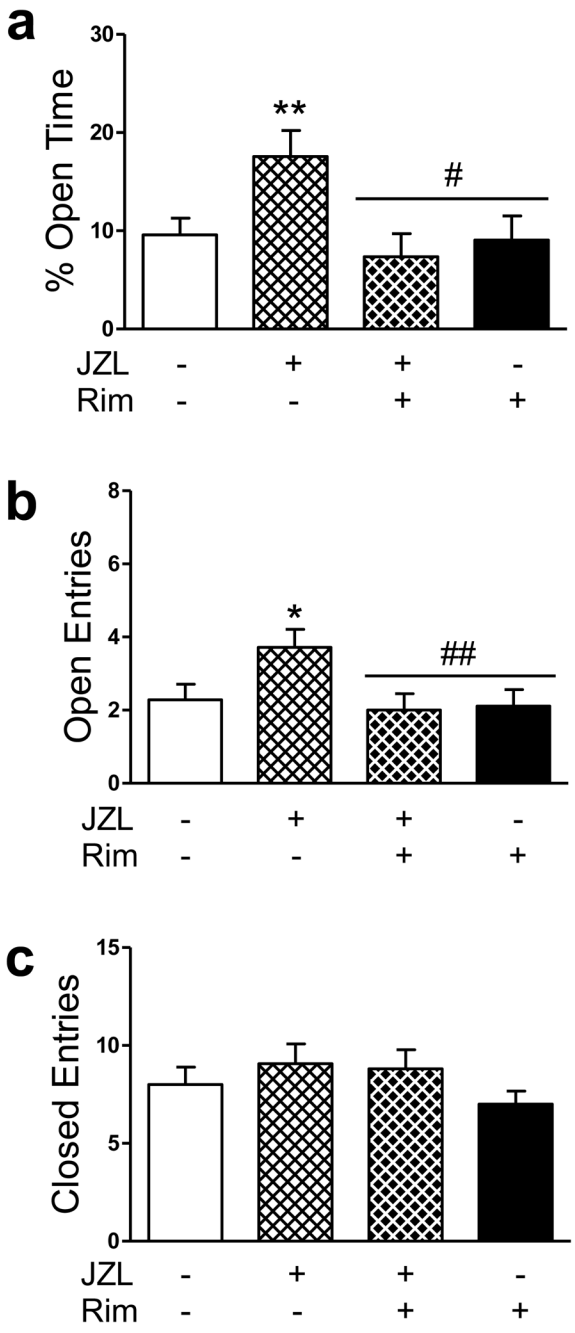

Figure 3. The CB_1 receptor antagonist rimonabant blocked the anxiolytic-like effects of JZL184 under conditions of high environmental aversiveness in the elevated plus maze

The increase in (a) percentage of open arm time and (b) number of open arm entries induced by JZL184 (JZL, 8 mg/kg i.p.) was blocked by the CB_1 receptor antagonist rimonabant (Rim, 1 mg/kg i.p.) under high light. Rats treated with either rimonabant (Rim, 1mg/kg i.p.) administered alone or in combination with JZL184 (JZL, 8 mg/kg i.p.) were pooled as they did not differ in (a) percentage of open arm time, or number of (b) open or (c) closed arm entries. Under high light, neither JZL184 (JZL, 8 mg/kg i.p.) nor rimonabant (Rim, 1 mg/kg i.p.), administered alone or in combination, altered (c) the frequency of closed arm entries,

relative to vehicle. Data are mean ± S.E.M (n = 9 – 14). $^{**}p < 0.01$, $^{*}p < 0.05$ vs. vehicle (*t*-test, one tailed); $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. JZL184 (*t*-test, one tailed).

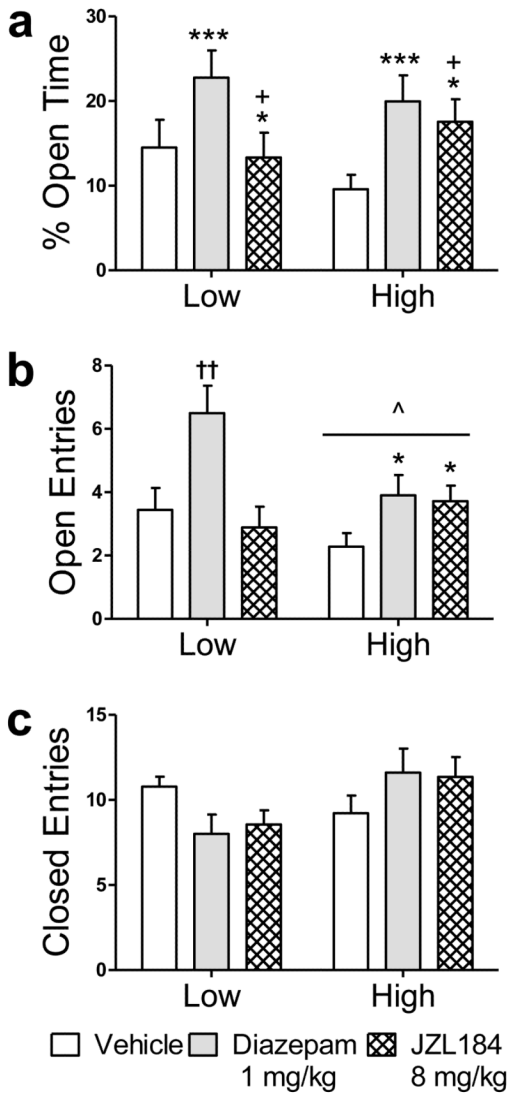

Figure 4. High environmental aversiveness produces anxiogenic-like responding in the elevated plus maze and unmasks anxiolytic-like effects of JZL184

Regardless of lighting condition, diazepam increased the (a) percentage of open arm time relative to vehicle. JZL184-treated rats exhibited (a) a lower percentage of open arm time and (b) fewer open arm entries relative to diazepam. High light itself reduced (b) the number of open arm entries without altering (a) the percentage of open arm time compared to low light. Under low light, diazepam increased the number of (b) open arm entries relative to this treatment under high light or vehicle or JZL184 under any light. Under high light, both JZL184 and diazepam increased the (b) number of open arm entries compared to vehicle. Neither drug treatment nor lighting condition altered (c) the frequency of closed arm entries. Data are mean ± S.E.M (n = 8 – 14). ***$p < 0.001$, *$p < 0.05$ vs. vehicle ([a]Group Main Effect or [b] Interaction Effect, LSD post hoc, one tailed), $^{+}p < 0.001$ vs. diazepam 1 mg/kg (Group Main Effect, LSD post hoc, one tailed), $^{††}p < 0.01$ vs. diazepam 1 mg/kg under low light (Interaction Effect, LSD post hoc, 1 tailed), $^{\wedge}p = 0.05$ vs. low light (Lighting Main Effect).

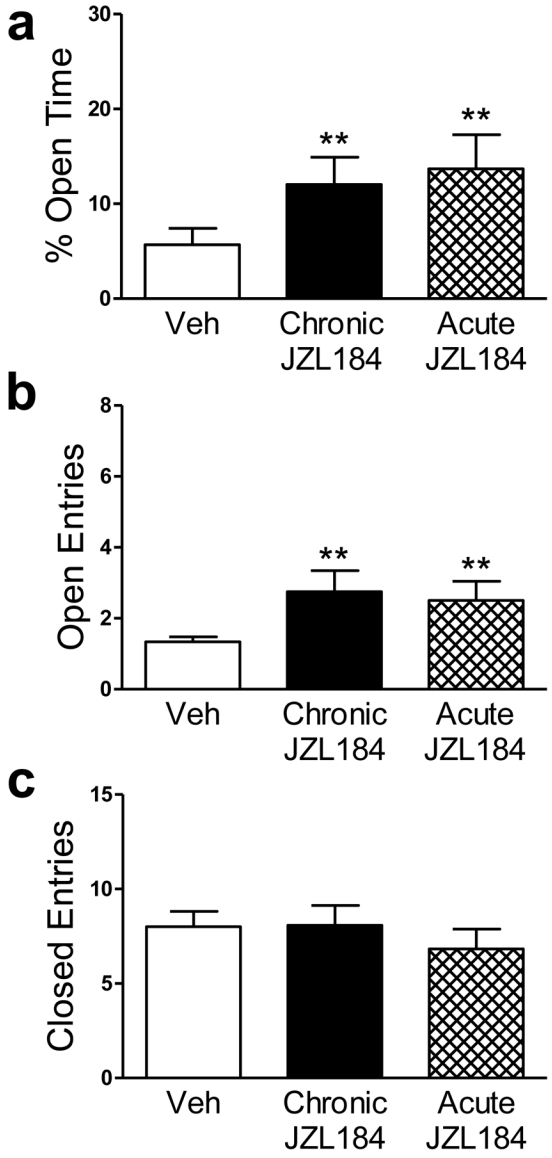

Figure 5. Chronic and acute administration of JZL184 produces anxiolytic-like responding in the elevated plus maze under conditions of high environmental aversiveness

Both chronic (8 mg/kg i.p. per day × 6 days) and acute (8 mg/kg i.p. on day 6 only) treatment with JZL184 increased the (a) percentage of open arm time and (b) number of open arm entries relative to vehicle-treated rats without altering the (c) number of closed arm entries. The (a) percentage of open arm time and (b) number of open arm entries did not differ between chronic and acute JZL184-treated groups. Data are mean ± S.E.M (n = 12). $^{**}p < 0.01$ vs. vehicle (*t*-test, one tailed).

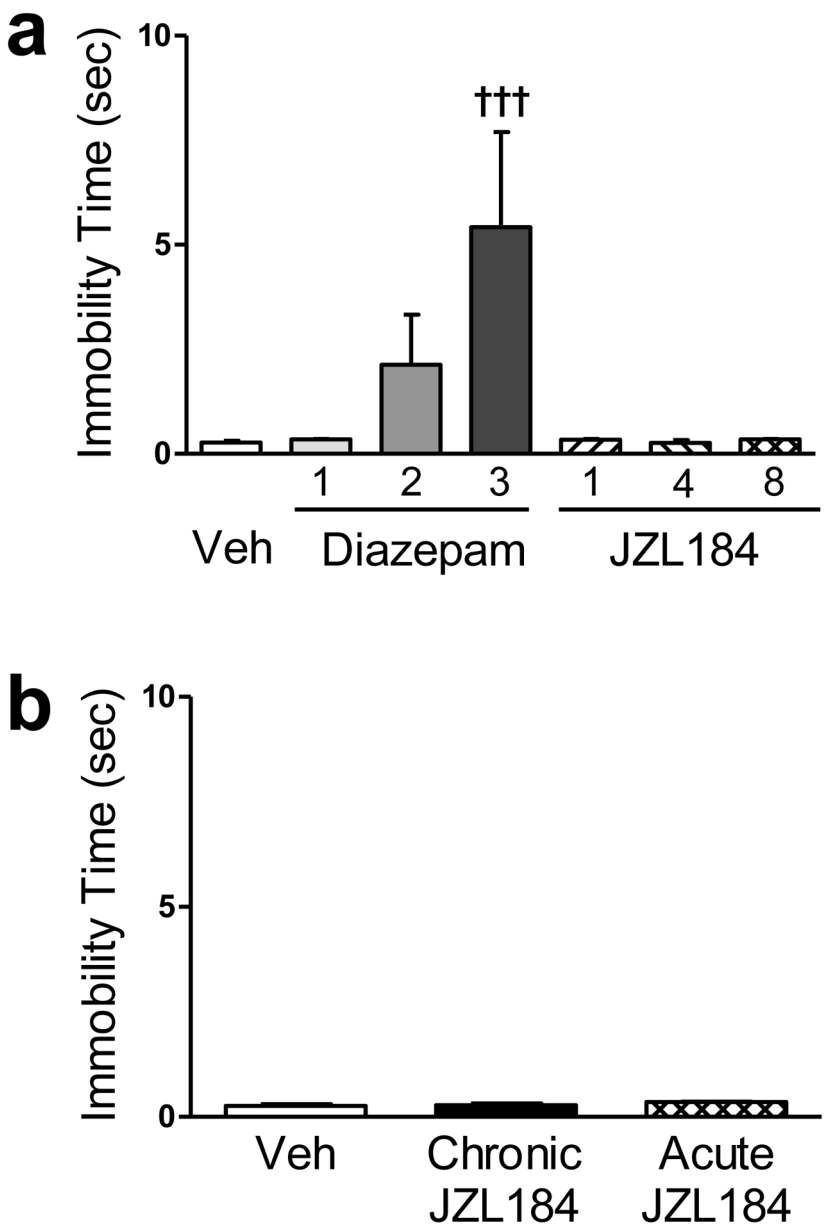

Figure 6. Diazepam, but not JZL184, produces catalepsy in the bar test

Acute administration of JZL184 (1, 4, 8 mg/kg) did not alter (a) immobility time in the bar test relative to vehicle. The high dose of diazepam (3 mg/kg) increased (a) immobility time in the bar test compared to vehicle, the low dose of diazepam (1 mg/kg), or all doses of JZL184 (1, 4, 8 mg/kg). The high dose (3 mg/kg) of diazepam was no different from the medium dose (2 mg/kg) in (a) time spent immobile in the bar test. Neither chronic (8 mg/kg i.p. × 6 days) nor acute (8 mg/kg i.p. on day 6 only) administration of JZL184 altered (b) immobility time in the bar test relative to vehicle. (b) Immobility time did not differ between rats receiving repeated injections that were treated either chronically or acutely with JZL184. Data are mean ± S.E.M (n = 5 – 14). $^{\dagger}p = 0.000$ vs. all other groups except diazepam 2 mg/kg (ANOVA, LSD post hoc, two tailed).

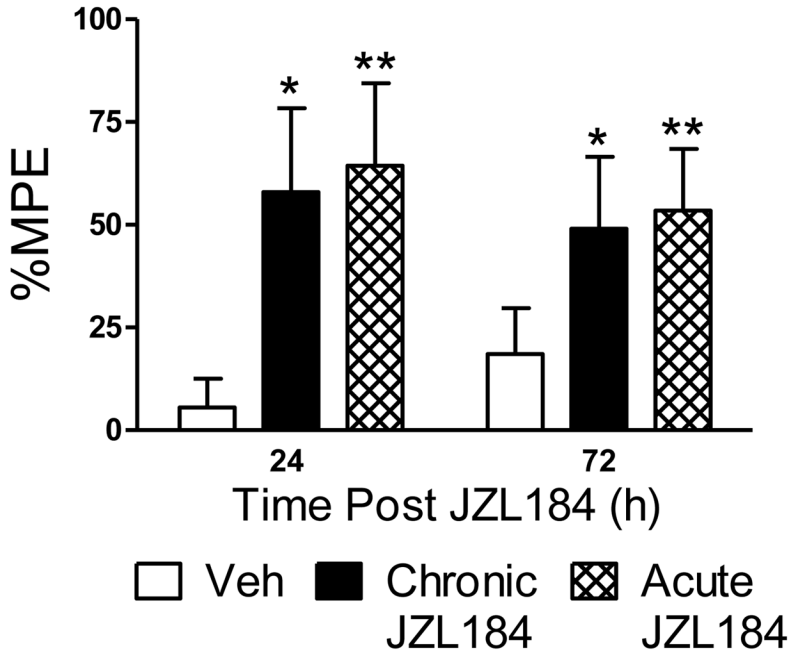

Figure 7. Chronic and acute treatment with JZL184 produces behavioral sensitization to exogenous cannabinoid administration

Both chronic (8 mg/kg i.p. per day × 6 days) and acute (8 mg/kg i.p. on day 6 only) treatment with JZL184 produced behavioral sensitization to antinociceptive effects of WIN55,212-2 (2.5 mg/kg i.p.). Antinociception was evaluated in the tail-flick test in the same rats used in the elevated plus maze test (see Fig. 6) either 24 or 72 h after the terminal injection of JZL184/vehicle. WIN55, 212-2-induced antinociception was greater in rats receiving JZL184 chronically (8 mg/kg per day × 6 days) or acutely (8 mg/kg i.p. on day 6 only) ($p = 0.006$; $\eta^2 = 0.282$) relative to chronic vehicle treatment. WIN55,212-2-induced antinociception did not differ between JZL184-treated groups. Data are mean ± S.E.M of % maximal antinociceptive effect (%MPE) (n = 6–12). *$p < 0.05$, **$p < 0.01$, vs. vehicle (ANOVA, LSD post hoc, one tailed).