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# Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling

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Running title: An anxiolytic role for endogenous cannabinoid signaling

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Number of
Text pages 26
Tables 0
Figures 7
References 37

Number of words in
Abstract 231
Introduction 747
Discussion 1443

Non-standard abbreviations: 2-AG: 2-arachidonylglycerol; AEA: *N*-arachidonylethanolamine; $CB_1$: cannabinoid receptor type 1; eCB: endocannabinoid; FAAH: fatty acid amide hydrolase; MGL: monoacylglycerol lipase; THC: $\Delta^9$-tetrahydrocannabinol; $T_o$: time spent on the open arms of the elevated plus maze.

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**ABSTRACT**

Extracts of *Cannabis sativa* have been utilized for their calming and sedative effects for centuries. Recent developments in drug discovery have suggested modulation of neuronal endogenous cannabinoid signaling systems could represent a novel approach to the treatment of anxiety-related disorders while minimizing the adverse effects of direct acting cannabinoid receptor agonists. In this study, we evaluated the effects of direct cannabinoid receptor agonists and antagonists; and endocannabinoid modulating drugs on anxiety-like behavior in mice using the elevated-plus maze. We found that the direct $CB_1$ receptor agonists CP 55940 (0.001-0.3 mg/kg) and Win 55212-2 (0.3-10 mg/kg) increased time spent on the open arms ($T_o$) at low doses only. At the highest doses tested, both compounds altered overall locomotor activity. In contrast, $\Delta^9$-tetrahydrocannabinol (0.25-10 mg/kg) produced a dose-dependent reduction in $T_o$. The endocannabinoid uptake/catabolism inhibitor AM404 (0.3-10 mg/kg) produced an increase in $T_o$ at low doses and had no effect at the highest dose tested. The fatty acid amide hydrolase inhibitor, URB597 (0.03-0.3 mg/kg) produced a monophasic, dose-dependent increase in $T_o$. The $CB_1$ receptor antagonists SR141716 (1-10 mg/kg) and AM251 (1-10 mg/kg) produced dose-related decreases in $T_o$. These data indicate that activation of $CB_1$ cannabinoid receptors reduces anxiety-like behaviors in mice and further support an anxiolytic role for endogenous cannabinoid signaling. These results suggest that pharmacological modulation of this system could represent a new approach to the treatment of anxiety-related psychiatric disorders.

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## INTRODUCTION

Marijuana is widely used throughout the world for recreational and therapeutic purposes (Adams and Martin, 1996). A common reason given for continued marijuana use in certain populations is reduction in anxiety and relaxation; however, adverse reactions including heightened anxiety and panic are common and widely cited reasons for discontinuation of marijuana use (Szuster et al., 1988; Thomas, 1996; Reilly et al., 1998). The adverse effects of marijuana are more pronounced during novel or stressful environmental conditions; after consumption of large doses of cannabis; and in naïve users (Abel, 1971; Gregg et al., 1976; Naliboff et al., 1976).

Animal data parallel these clinical observations. Direct agonists of the cannabinoid receptor type-1 ($CB_1$), the molecular target of the principal psychoactive component of marijuana, $\Delta^9$-tetrahydrocannabinol (THC), produce bi-phasic effects on anxiety. Relatively low doses produce anxiolytic-like effects in animals, while higher doses produce an anxiogenic profile (see (Viveros et al., 2005) for review). In addition, prior exposure of animals to stress sensitizes them to the anxiogenic effects of cannabinoid agonists and stress interacts synergistically with cannabinoid agonists to activate stress-related brain regions including the central amygdala (Hill and Gorzalka, 2004; Patel et al., 2005b). While these data suggest that appropriate modulation of cannabinoid systems could have therapeutic potential in the treatment of anxiety-related neuropsychiatric disorders; the biphasic and context-dependent effects of direct $CB_1$ receptor agonists limit their therapeutic potential.

Recent data suggest that pharmacological agents that enhance endogenous cannabinoid (eCB) signaling provide a novel approach to the treatment of anxiety-related

disorders. The identification and characterization of the molecular substrates of eCB signaling have led to the development of pharmacological agents that augment eCB signaling. ECBs, which include *N*-arachidonylethanolamine (AEA) and 2-arachidonylglycerol (2-AG), are synthesized by neurons via independent enzymatic cascades. After release, AEA and 2-AG and are thought to be recaptured by neurons and degraded by fatty acid amide hydrolase (FAAH) and monacylglycerol lipase (MGL), respectively (see (Piomelli, 2003) for review). Activation of presynaptic $CB_1$ receptors leads to a reduction in neurotransmitter release, and eCB signaling likely plays a role in retrograde inhibition of neurotransmitter release in many brain regions. The localization of eCBs, $CB_1$ receptors, FAAH, and MGL within brain regions subserving emotion and motivation (i.e. the prefrontal cortex, nucleus accumbens, amygdala, and hypothalamus (Tsou et al., 1998a; Tsou et al., 1998b; Marsicano and Lutz, 1999; Katona et al., 2001; McDonald and Mascagni, 2001; Gulyas et al., 2004)) is also consistent with a role for this system in the regulation of emotional behavior.

Specifically, brain AEA content is significantly increased by the FAAH inhibitor, URB597 (Kathuria et al., 2003). URB597 reduces anxiety-like behaviors in the elevated zero maze and isolation-induced ultrasonic vocalizations in pups (Kathuria et al., 2003); and reduces restraint stress-induced corticosterone release (Patel et al., 2004); all with linear dose-response relationships. In contrast to direct $CB_1$ receptor agonists, URB597 does not induce place preference in rodents suggesting it could be devoid of the abuse potential inherent in direct $CB_1$ agonists (Gobbi et al., 2005). Another target for the development of indirect agonists of the $CB_1$ receptor is the eCB transport/uptake process since inhibition of neuronal accumulation of the eCBs increases availability of eCBs

AEA and 2-AG at $CB_1$ receptors (Hajos et al., 2004). AM404 is an inhibitor of eCB uptake and an inhibitor of FAAH (Jarrahian et al., 2000). AM404 reduces restraint-induced corticosterone release in a biphasic manner, with only low doses producing a reduction in corticosterone release (Patel et al., 2004). Thus, the dose-response properties of AM404 are more like those of the direct $CB_1$ agonist CP55940 than the FAAH inhibitor URB597. In summary, URB597 appears to be devoid the biphasic properties of direct $CB_1$ agonists and AM404 (Kathuria et al., 2003; Patel et al., 2004; Gobbi et al., 2005), and does not interact with environmental stress to potentiate activation of the central amygdala (Patel et al., 2005b). For these reasons, selective inhibitors of FAAH could be viable alternatives to direct $CB_1$ agonists for the development of cannabinoid based anti-anxiety treatments.

Although numerous studies exploring the effects of exogenous cannabinoids and eCB signaling in the modulation of anxiety have been published, a comparative data using a variety of ligands and dose ranges within the same laboratory have not been compiled. Thus, the purpose of these studies was to evaluate the effects of direct and indirect $CB_1$ receptor agonists, and $CB_1$ antagonists on anxiety behaviors using the elevated-plus maze paradigm in mice. We utilized wide dose ranges and multiple drugs from each class in order to clarify the role of eCB signaling in the regulation of anxiety-related behaviors.

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## METHODS

### Animals and drugs

All experiments were carried out in accordance with the NIH Guide for the Use and Care of Laboratory Animals. ICR male mice (21-24 g) were used in all experiments (Harlan, Madison WI). All animals were housed on a 12:12 light/dark cycle with lights on at 6:00. Animals had *ad lib* access to food and water. All experiments were conducted between 09:00 and 12:00. Each animal was used only once; approximately 300 mice were used in this study.

AM404 (4-hydroxyphenylarachidonylamide) and AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) were purchased from Tocris Cookson (Ellisville, MO). URB597 (cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester) was purchased from Cayman Chemical (Ann Arbor, MI). SR141716 (rimonabant) was a gift from the NIDA Drug-Supply Program (Research Triangle Park, NC). CP55940 (5-(1,1-dimethylheptyl)-2-[5-hydroxypropyl)cyclohexyl]phenol) was a gift from Pfizer Central Research (Groton, CT). (R)-(+)Win 55212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo [1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate) was purchased from Sigma Chemical (St. Louis, MO).

All drugs except URB597 were dissolved in ethanol-emulphor vehicle (18:1:1, saline/emulphor/ethanol); URB597 was dissolved in DMSO-emulphor vehicle (18:1:1 saline/emulphor/dimethylsulfoxide). All drugs were delivered by i.p. injection 30 min

prior to experimentation in a volume of 10 ml/kg. Each experimental group (i.e. the mice included in a particular session) included vehicle treated mice.

**Elevated-plus maze**

For each treatment group, 8-10 animals were used. Animals were habituated to the testing room for at least 2 h prior to experimentation. The elevated-plus maze (San Diego Instruments, San Diego, CA) was constructed of beige ABS plastic and consisted of two open arms (30 cm x 5 cm) and two enclosed arms (30 cm x 5 cm x 15 cm) that extended from a central platform (5 cm x 5 cm). The maze was elevated 40 cm above the floor. Experiments began by placing a single mouse on the central platform facing an open arm. During the first 5 min of free exploration, the number of entries into (defined as an animal placing all four paws onto an arm), and time spent in open and closed arms were recorded by a trained observer blind to treatment condition. The maze was cleaned thoroughly between animals using a 50% ethanol solution.

**Statistical analysis**

Percent time spent in open arm exploration ([time spent in open arms/time spent in open arms + time spent in closed arms] X 100), absolute time spent in open arm exploration, and percent open arm entries ([number of open arm entries/number of open arm entries + number of closed arm entries] X 100) were considered measures of anxiety; total arm entries provided a measure of overall locomotor activity. Between group comparisons were conducted by one-way ANOVA followed by Dunnett's test; $p<0.05$ was considered significant throughout.

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## RESULTS

### Effects of $CB_1$ receptor agonists on anxiety-like behaviors

The high efficacy $CB_1$ receptor agonist, CP55940, significantly increased the percent time spent in open arm exploration at 0.01, 0.03, and 0.3 mg/kg compared to vehicle treatment, while 0.1 mg/kg was not significantly different from control (Fig. 1). CP55940 significantly increased time spent in open arm exploration, percent open arm entries, and number of total arm entries at 0.3 mg/kg only. At the 0.3 mg/kg dose of CP55940, mice exhibited a stereotypic behavior characterized by repetitive and rapid entries into open arms. Given the large increase in overall activity at this dose, the validity of this test as a measure of anxiety is likely compromised.

A second, high efficacy $CB_1$ receptor agonist, Win 55212-2 significantly increased the percent time spent in open arm exploration at 1 and 3 mg/kg compared to vehicle treatment, while 10 mg/kg was not significantly different from control (Fig. 2). Win 55212-2 also significantly increased time spent in open arm exploration and percent open arm entries at 1 and 3 mg/kg compared to vehicle treatment, while 10 mg/kg was not significantly different from control on either measure. Win 55212-2 significantly increased the total number of arm entries at 3 mg/kg and significantly decreased the total number of arm entries at 10 mg/kg, thus compromising the validity of this test as a measure of anxiety at these doses.

THC, at doses of 1, 2.5 and 10 mg/kg, significantly decreased percent time spent in open arm exploration (Fig. 3). THC significantly reduced time spent in open arm

exploration and percent open arm entries at 2.5 and 10 mg/kg. THC did not affect the total number of arm entries at any dose tested.

**Effects of $CB_1$ receptor antagonists on anxiety-like behaviors**

To determine whether tonic eCB signaling affects anxiety-like behaviors, we assessed the effects of the $CB_1$ receptor antagonists AM251 and SR141716 on elevated-plus maze performance in male mice. AM251 significantly decreased the percent time spent in open arm exploration at 3 and 10 mg/kg compared to vehicle treatment (Fig. 4). AM251 also decreased the time spent in open arm exploration at 3 and 10 mg/kg, and decreased the percent open arm entries at 10 mg/kg. AM251 did not significantly affect the total number of arm entries at any dose examined.

SR141716 significantly reduced the percent time spent in open arm exploration at doses of 3 and 10 mg/kg (Fig. 5). At 10 mg/kg, SR141716 produced a significant reduction in the time spent in open arm exploration. SR141716 did not significantly effect the percent open arm entries, or the total number of arm entries, at any dose tested.

**Effects of eCB modulators on anxiety-like behaviors**

To determine whether pharmacological augmentation of eCB signaling has effects similar to direct $CB_1$ receptor activation, we assessed the effects of eCB modulators URB597 and AM404 on anxiety-like behaviors using the elevated-plus maze. URB597 significantly increased the percent time spent in open arm exploration at 0.1 and 0.3 mg/kg (Fig. 6). URB597 also significantly increased the time spent in open arm

exploration and the percent of open arm entries at 0.1 mg/kg. URB597 did not affect the total number of arm entries at any dose tested.

AM404 significantly increased the percent time spent in open arm exploration at 1 and 3 mg/kg, while a dose of 10 mg/kg was not different from control (Fig. 7). AM404 also significantly increased the time spent in open arm exploration at 3 mg/kg with a dose of 10 mg/kg producing no effect compared to control. AM404 did not significantly increase the percent of open arm entries or affect the total number of arm entries at any dose tested.

## DISCUSSION

The laboratory study of the effects of cannabinoids on anxiety-like behaviors in rodents has been hampered by some unique properties of the cannabinoids compared to classic anxiolytics such as benzodiazepines. Specifically, effects of cannabinoids on anxiety-like behaviors are dependent on the pharmacological properties and selectivity of the available ligands; dose; species and strain; basal and previous stress exposure; and possibly other variables (see (Viveros et al., 2005) for discussion of these issues). Although many previous studies have explored the role of various cannabinoid ligands on anxiety-like behavior using the elevated-plus maze, the lack of multiple ligand and dose comparisons combined with the lack of comparability between studies (based on the reasons cited above), have hampered the development of a clear picture of the effects of cannabinoids on anxiety. The purpose of this study was to compare broad dose ranges of several structurally-diverse direct $CB_1$ cannabinoid receptor agonists, two $CB_1$ antagonists, as well as eCB modulating compounds, to gain insight into the role of $CB_1$ receptor signaling in the physiological modulation of anxiety.

We evaluated three direct acting $CB_1$ receptor agonists: CP55940, Win 55212-2, and THC; on the behavior of mice on the elevated-plus maze. The dose response relationship for CP55940 is complex. Between 1 and 30 µg/kg, CP55940 produced a dose-related increase in behavior that is consistent with a reduction in the fearfulness or anxiety of the mice. However, at a dose of 100 µg/kg, this trend was reversed completely and no significant differences from vehicle were seen. When the dose was increased further, stereotypic behaviors were observed and a significant increase in the total number of arm entries was seen. This change in behavior, which likely reflects a large

increase in overall locomotor activity, compromises the validity of the behavioral assay as a measure of anxiety at this dose. Therefore, these data indicate that at doses of less than 30 µg/kg, CP55940 produces anxiolytic-like effects in mice; however, doses of 100 µg/kg or higher are either ineffective or produce data that cannot be interpreted due to locomotor effects. A somewhat similar pattern of effects were observed with Win 55212-2. A relatively low dose (1 mg/kg) produced increased open arm entries and time on the open arms compared to vehicle while the effects of doses of 3 mg/kg and higher were confounded by changes in total arm entries. These data are generally consistent with previous findings in mice and rats that low doses of high efficacy $CB_1$ agonists produce anxiolytic effects, while higher doses have no effect or are possibly anxiogenic (Haller et al., 2004; Marco et al., 2004).

In contrast, the low efficacy $CB_1$ agonist THC, displayed a linear, dose-dependant anxiogenic effect similar to the findings of Onaivi and co-workers (Onaivi et al., 1990). Although the reasons for the differences between CP55940/Win 55212-2 and THC are not clear, one likely contribution is the difference in efficacy among these agonists. Both CP 55940 and Win 55212-2 exhibit high efficacy for $CB_1$ receptor activation while THC is quite low in comparison (approximately 10% of Win 55212-2; (Kearn et al., 1999)). In fact, when GDP/GTP exchange is used as an assay for efficacy, THC has lower efficacy than the endocannabinoids AEA and 2-AG (Kearn et al., 1999; Hillard, 2000). As a result, THC will act as an antagonist at $CB_1$ receptors that have high occupancy by endogenous ligands. This possibility is supported by our data that the $CB_1$ receptor antagonists SR141716 and AM251 also produce anxiogenic effects. Other studies have also concluded that THC can act as a physiological antagonist under certain conditions

(Kelley and Thayer, 2004; Straiker and Mackie, 2005). In light of recent data that $CB_1$ receptor agonists can differentially direct signaling to specific sub-types of G-proteins within specific cells, it is also possible that the three agonists studied induce different signaling pathways even though they activate the same receptor (Mukhopadhyay and Howlett, 2005).

Our finding that THC does not produce anxiolytic-like effects in this model is interesting in light of the anxiolytic and calming subjective effects of cannabis intoxication in humans. One explanation is that *Cannabis sativa* contains over 100 distinct compounds (Brenneisen and elSohly, 1988), some of which could be anxiolytic by $CB_1$ or non–$CB_1$ receptor-dependent mechanisms. In support of this possibility, we, and others have shown that cannabidiol, a compound found in *Cannabis sativa* preparations that does not interact with $CB_1$ receptors, exerts robust anxiolytic effects in mice via an unknown mechanism (S.P. and C.J.H unpublished data; (Guimaraes et al., 1994)). It is possible that other compounds found in plant preparations could act together with THC in producing subjective experiences that occur following cannabis use. This notion is not supported, however, by data indicating only minor differences between the subjective effects of pure THC and marijuana in humans (Hart et al., 2002; Wachtel et al., 2002).

We also evaluated the effects of two $CB_1$ receptor antagonists, AM251 and SR141716, on the behavior of mice in the elevated-plus maze. Both compounds reduce the time spent on the open arms and therefore, exhibit anxiogenic-like effects in mice. These findings are consistent with some previous reports (Navarro et al., 1997; Arevalo et al., 2001; Haller et al., 2004). However, in another study, SR141716 was found to

produce an anxiolytic effect that was also observed in $CB_1^{-/-}$ mice, indicating that this effect was not mediated by $CB_1$ receptors (Haller et al., 2002). However, AM251 had no effect on the activity of $CB_1^{-/-}$ mice in the elevated-plus maze, suggesting that the alternative mechanism is selective for SR141716 (Haller et al., 2004). In addition, acute SR141716 administration in rats also produces anxiolytic effects (Griebel et al., 2005). These authors suggest that the basal level of anxiety determines the net effect of SR141716 on anxiety-like behaviors. This is supported by data demonstrating that an anxiolytic effect of SR141716 is revealed in mice previously exposed to the maze apparatus (Rodgers et al., 2003). Since previous exposure to the maze decreased the time spent in open arm exploration (i.e. produced an anxiogenic profile), the anxiolytic effects of SR141716 are more pronounced when animals have increased basal levels of anxiety. However, in contrast to these previous studies, we failed to reveal any anxiolytic effects of SR141716, although it does appear that AM251 is more potent that SR141716 in producing anxiogenic effects in male ICR mice. Taken together, our present results as well as those of others (Kathuria et al., 2003; Haller et al., 2004; Patel et al., 2004) indicate that global activation of $CB_1$ receptor signaling shifts the internal state of mice toward a reduction in fearfulness and increased exploration. However, it is also clear that this effect is dose-sensitive, suggesting that $CB_1$ receptor activation is not purely "anti-anxiety".

To further evaluate the role of eCB signaling in the modulation of anxiety, we determined the effects of eCB modulating compounds URB597 and AM404 on elevated-plus maze performance in mice. URB597, a compound that increases brain AEA content (as well as other *N*-acylethanolamines, including *N*-palmitoylethanolamine and *N*-

oleylethanolamine) via inhibition of FAAH activity (Kathuria et al., 2003; Patel et al., 2005a), produced a linear, dose-dependent anxiolytic effect. This finding replicates data by Piomelli and co-workers (Kathuria et al., 2003) demonstrating an linear, dose-dependent anxiolytic effect of this compound in the elevated-zero maze in rats. Although it is possible that higher doses of URB597 could convert the dose-response curve from monophasic to U-shaped, the highest dose used (0.3 mg/kg) has been shown previously to produce maximal FAAH inhibition and increased AEA content beginning 15 minutes after administration (Kathuria et al., 2003). Furthermore, higher doses could reduce the selectivity of this compound for FAAH relative to other serine hydrolase enzymes. Interestingly, AM404, like direct acting $CB_1$ agonists, produces a biphasic effect on anxiety with low doses producing anxiolytic effects and the highest dose having no effect. AM404 is thought to exert its actions by increasing the availability of both AEA and 2-AG for $CB_1$ receptors by inhibiting their re-uptake and/or degradation (Freund et al., 2003). These findings suggest selective augmentation of AEA signaling may be preferred over the non-selective enhancement of AEA and 2-AG signaling, as a novel approach to developing anti-anxiety pharmacotherapeutics. Similar profiles for these compounds was demonstrated with regard to inhibition of stress-induced corticosterone release. URB597 produced linear, dose-dependent inhibition of stress-induced corticosterone release, where as AM404 produced a biphasic effect (Patel et al., 2004). We have previously shown that acute restraint stress decreases AEA content within the amygdala and forebrain, thus normalization of brain AEA signaling by FAAH inhibitors or eCB transport inhibitors during stress could underlie the anxiolytic properties of these compounds (Patel et al., 2005c).

Lastly, although many of the effects of URB597 and AM404 on anxiety-related behaviors are blocked by $CB_1$ receptor antagonists (Kathuria et al., 2003; Patel et al., 2004; Hill and Gorzalka, 2005), these compounds could exert effects on behavior independent of $CB_1$ receptor activation. For example, URB597 increases brain content of *N*-oleoylethanolamine which can activate peroxisome-proliferator-activated receptor-alpha (Fu et al., 2003). We are currently testing the hypothesis that the effects of URB597 and AM404 are mediated via $CB_1$ receptors by determining their effects on anxiety behaviors in $CB_1^{-/-}$ mice.

In summary, we have evaluated the effects of broad dose ranges of multiple direct cannabinoid receptor agonists, antagonists, and eCB modulating compounds on anxiety-like behaviors in mice using the elevated-plus maze. We conclude that eCB signaling is part of an endogenous anxiolytic neuromodulatory system, and that inhibition of FAAH activity represents a viable and promising approach to the development of treatments for anxiety-related psychiatric disorders. Preclinical data suggest that these compounds will avoid the adverse effects of direct $CB_1$ agonists including abuse potential and biphasic dose-response relationships.

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**FOOTNOTES**

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**FIGURE LEGENDS**

Figure 1. Effects of CP55940 on % open time (a), absolute open time (b), % open arm entries (c), and number of total arm entries (d) during a 5 minute exposure to the elevated-plus maze. *p<0.05, **p<0.01; significantly different from vehicle control.

Figure 2. Effects of Win 55212-2 on % open time (a), absolute open time (b), % open arm entries (c), and number of total arm entries (d) during a 5 minute exposure to the elevated-plus maze. *p<0.05, **p<0.01; significantly different from vehicle control.

Figure 3. Effects of THC on % open time (a), absolute open time (b), % open arm entries (c), and number of total arm entries (d) during a 5 minute exposure to the elevated-plus maze. *p<0.05, **p<0.01; significantly different from vehicle control.

Figure 4. Effects of AM251 on % open time (a), absolute open time (b), % open arm entries (c), and number of total arm entries (d) during a 5 minute exposure to the elevated-plus maze. **p<0.01; significantly different from vehicle control.

Figure 5. Effects of SR141716 on % open time (a), absolute open time (b), % open arm entries (c), and number of total arm entries (d) during a 5 minute exposure to the elevated-plus maze. *p<0.05; significantly different from vehicle control.

Figure 6. Effects of URB 597 on % open time (a), absolute open time (b), % open arm entries (c), and number of total arm entries (d) during a 5 minute exposure to the elevated-plus maze. *$p<0.05$, **$p<0.01$; significantly different from vehicle control.

Figure 7. Effects of AM404 on % open time (a), absolute open time (b), % open arm entries (c), and number of total arm entries (d) during a 5 minute exposure to the elevated-plus maze. *$p<0.05$, **$p<0.01$; significantly different from vehicle control.

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Figure 1

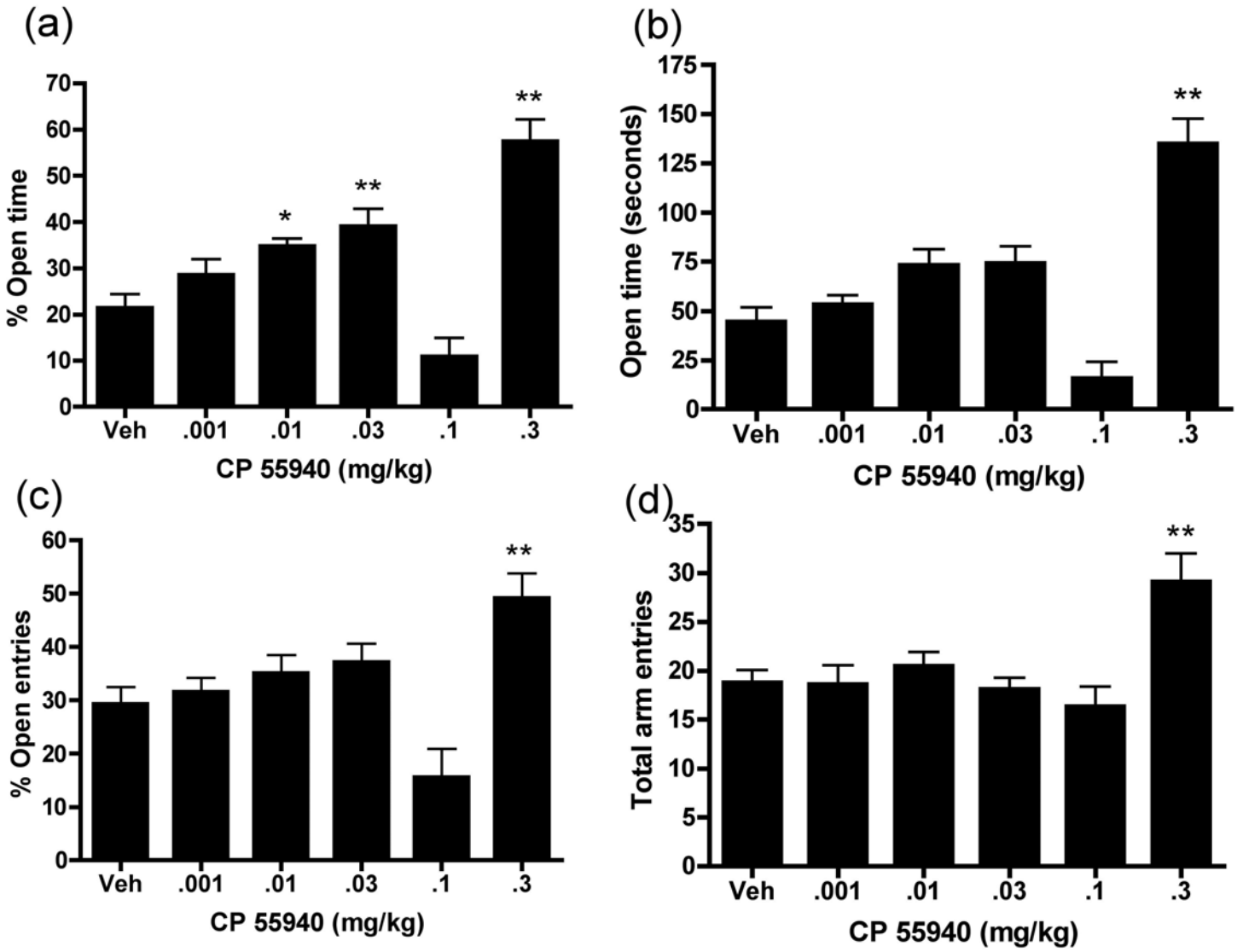

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Figure 2

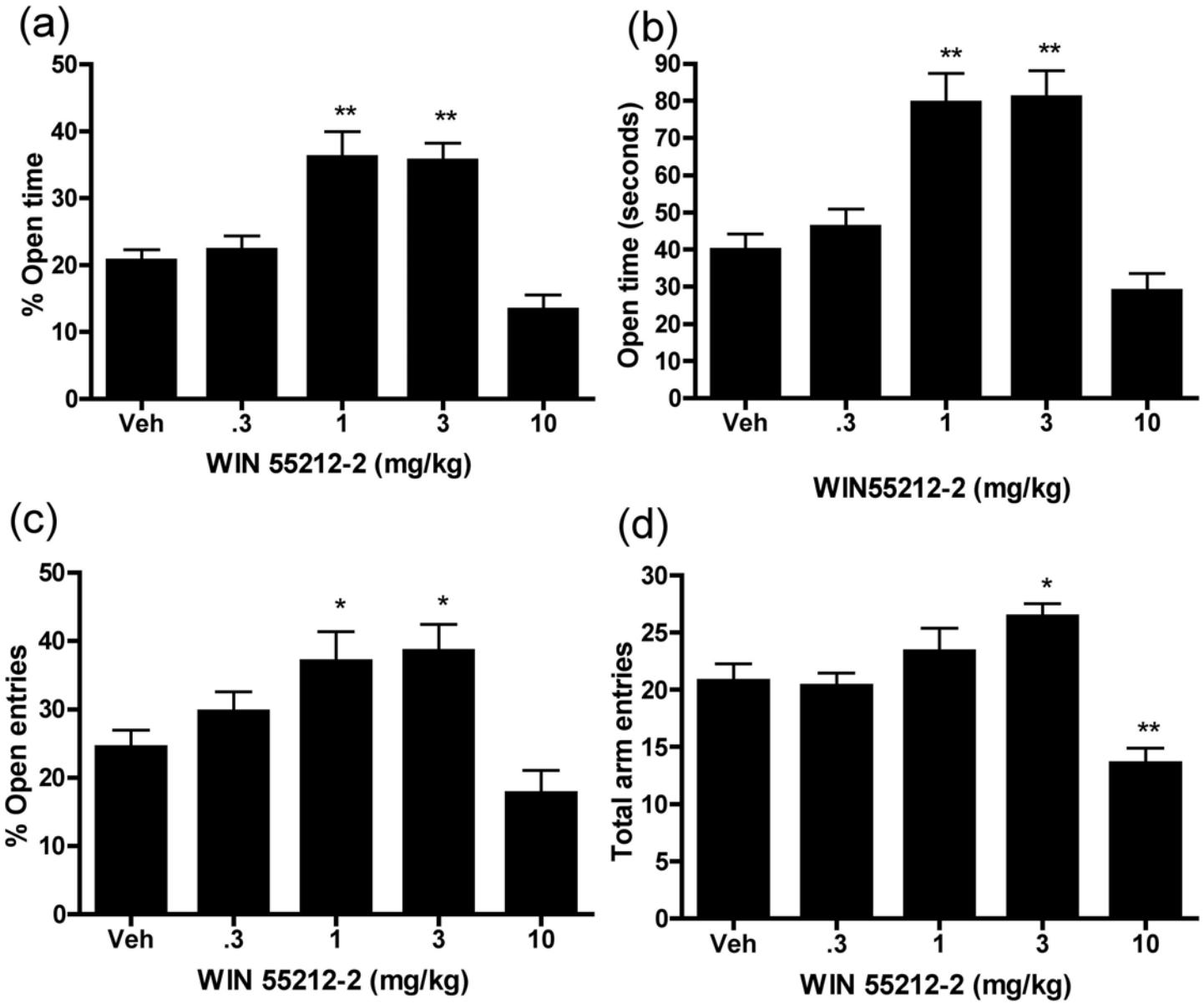

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Figure 3

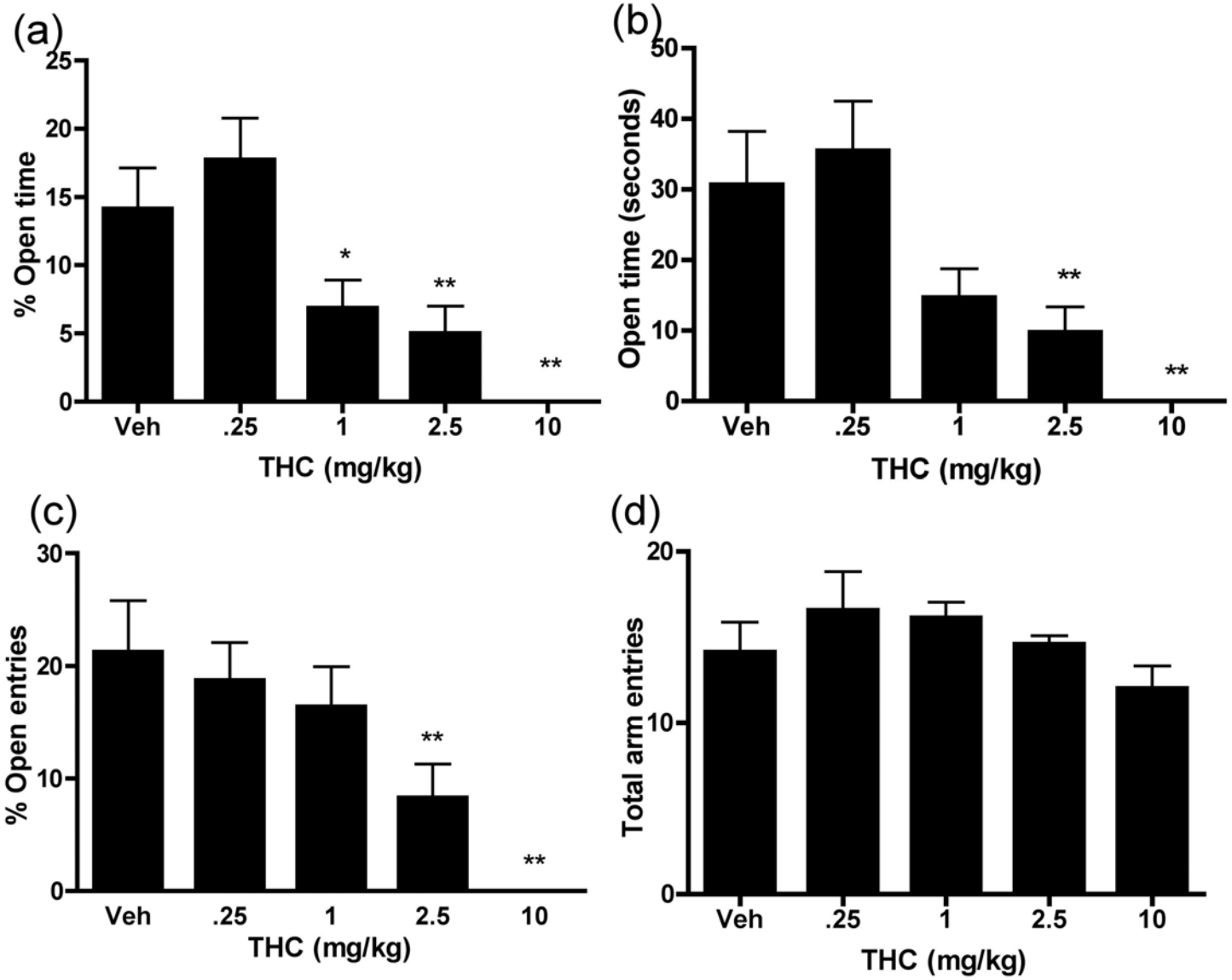

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Figure 4

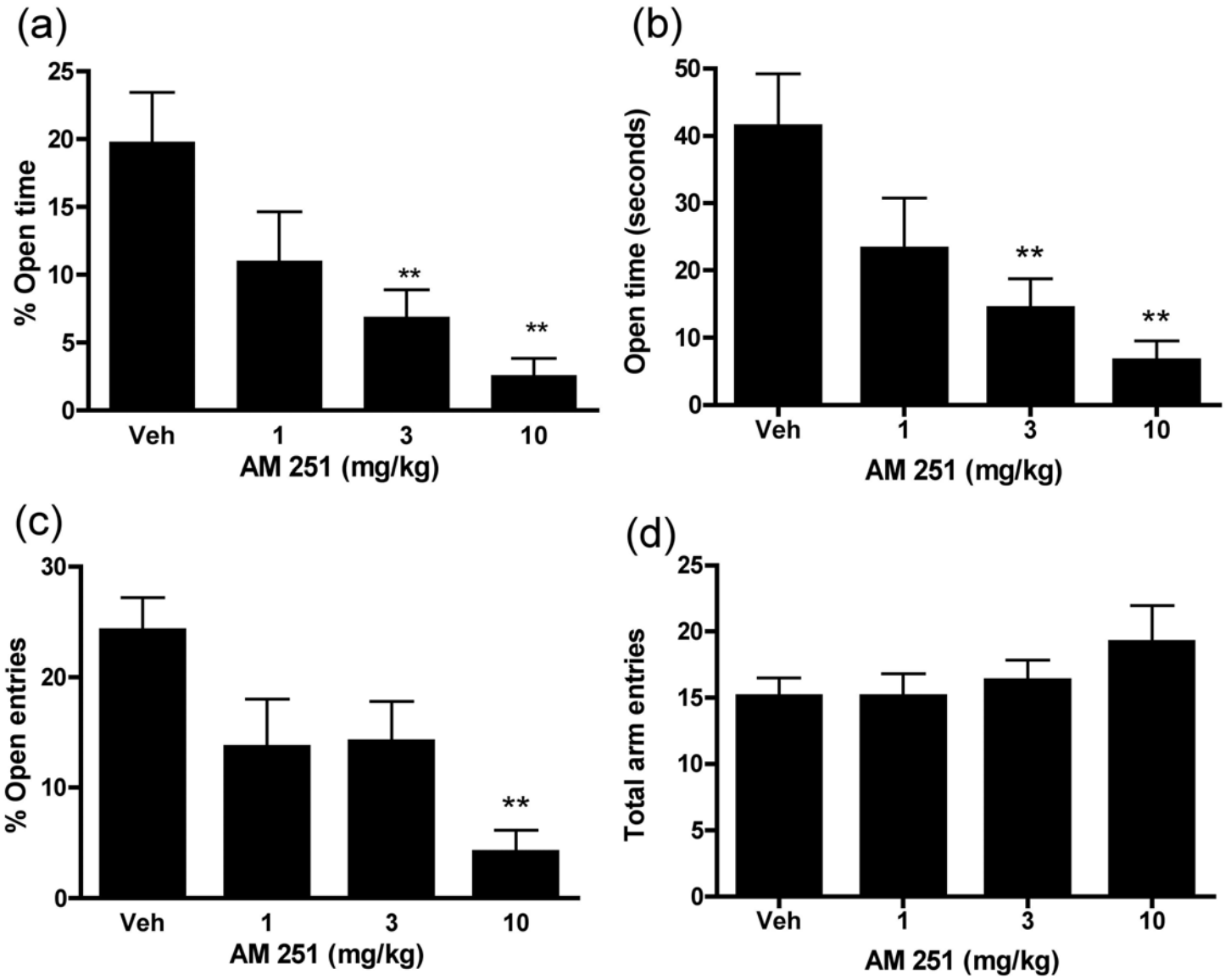

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Figure 5

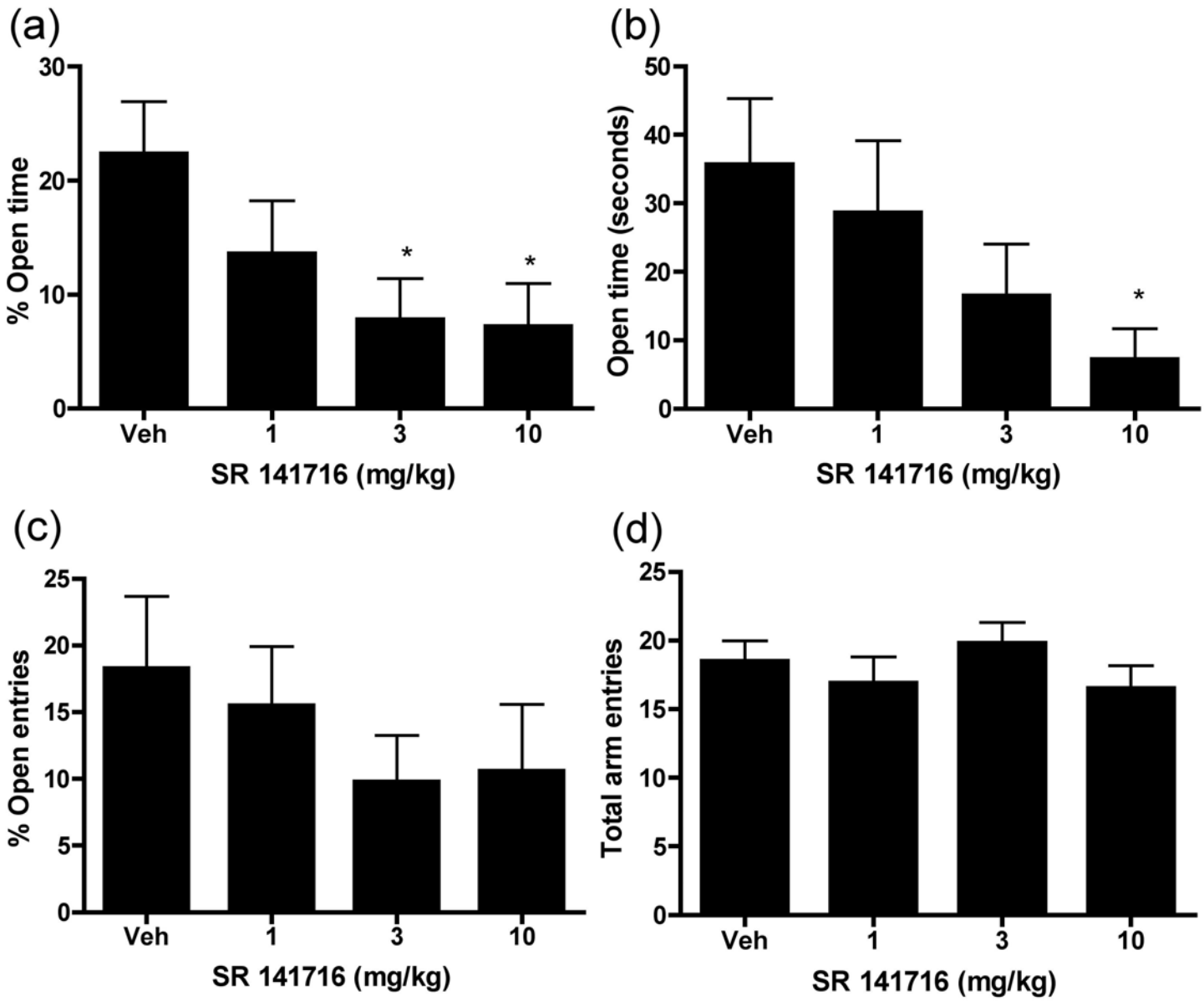

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Figure 6

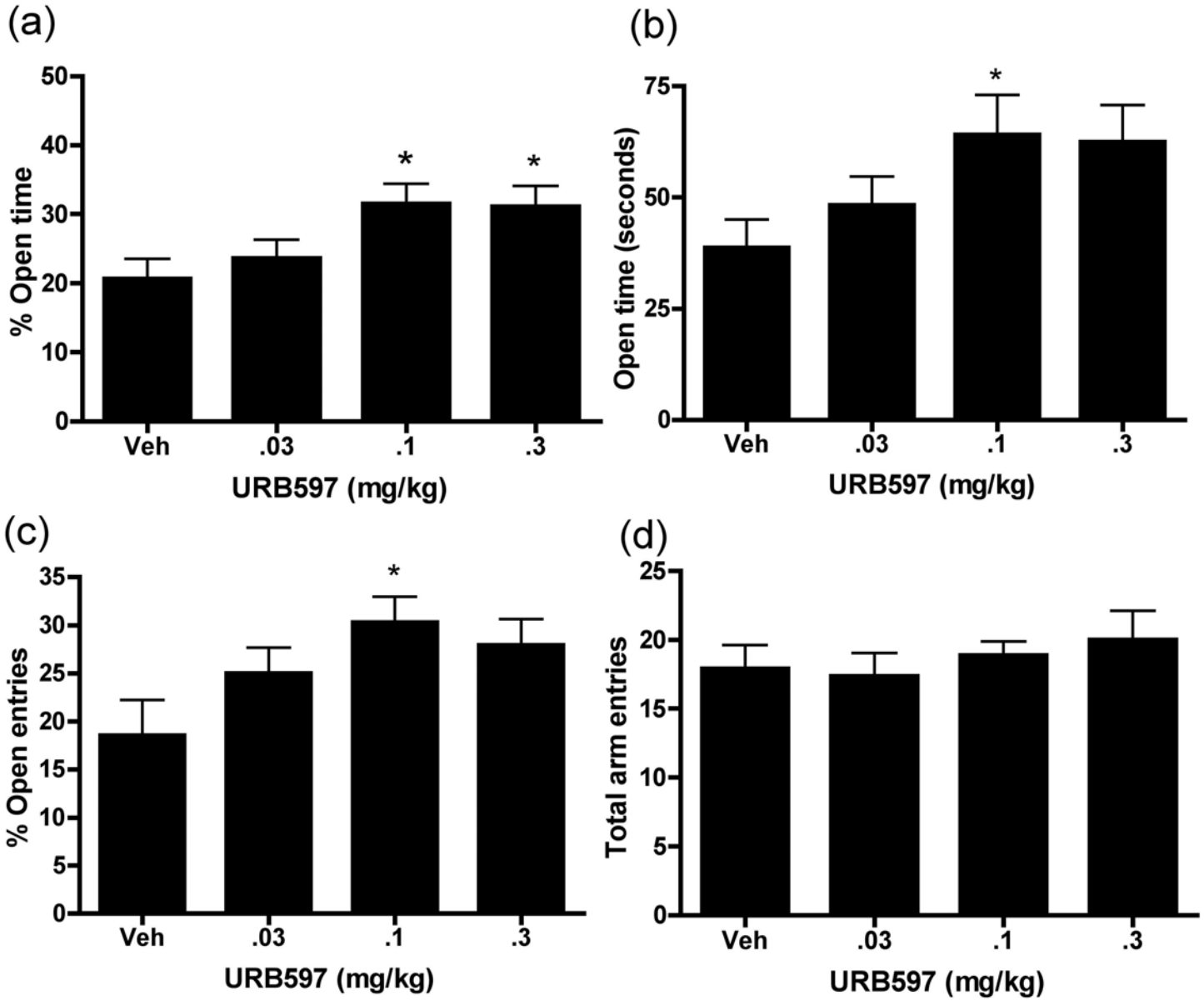

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Figure 7