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Cannabinoid-induced upregulation of serotonin 2A receptors in the hypothalamic paraventricular nucleus and anxiety-like behaviors in rats

Jade M. Franklin¹, Matt Mathew¹, and Gonzalo A. Carrasco^{1,*}

¹Department of Pharmacology and Toxicology, School of Pharmacy, University of Kansas, 1251 Wescoe Hall Drive, 3048B Malott Hall, Lawrence, KS 66045

Abstract

Recent behavioral reports suggest that repeated exposure to cannabis and synthetic cannabinoid agonists is linked with mental disorders associated with dysfunction of serotonin 2A (5-HT_{2A}) receptor neurotransmission such as anxiety and depression. Here, we studied the effect of a nonselective cannabinoid agonist, CP55940, on the activity of 5-HT_{2A} receptors in hypothalamic paraventricular nucleus (PVN). We detected that repeated exposure to CP55940 enhanced the prolactin and corticosterone neuroendocrine responses mediated by 5-HT_{2A} receptors and increased the membrane-associated levels of 5-HT_{2A} receptors in PVN. Importantly, we also detected increased anxiety-like behaviors in CP55940 treated rats compared to controls. The data presented here suggest that the mechanisms mediating the cannabinoid-induced upregulation of 5-HT_{2A} receptors would be brain-region specific, as we were unable to detect a CP55940-induced upregulation of 5-HT_{2A} mRNA. Our results might provide insight into the molecular mechanism by which repeated exposure to cannabinoids could be associated with the pathophysiology of neuropsychiatric disorders.

Keywords

marijuana; serotonin; hypothalamus; 5-HT_{2A} receptor; G-proteins

1. Introduction

A number of recent behavioral studies suggest that administration of Δ^9 -Tetrahydrocannabinol (Δ^9 -THC), the main psychoactive component of marijuana, or several synthetic cannabinoids can regulate the activity of serotonin 2A (5-HT_{2A}) receptors [11;24]. While acute cannabinoid administration reduces 5-HT_{2A} receptor-mediated behavioral responses [11]; repeated exposure to cannabinoids seems to be associated with increased behavioral responses to 5-HT_{2A} receptor agonists in adult rats [24]. Accordingly, we have recently reported that repeated exposure to cannabinoid agonists upregulates and

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*Correspondence: Gonzalo A. Carrasco, PhD, Department of Pharmacology and Toxicology, University of Kansas, School of Pharmacy, 1251 Wescoe Hall Drive, 3048B Malott Hall, Lawrence, KS 66045, Phone: 785-864-1974, Fax: 785-864-5219, carrasco@ku.edu.

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increases the activity of 5-HT_{2A} receptors in rat prefrontal cortex (PFCx) and in two neuronal cell models [17–20].

Cannabinoids produce their responses by activating two cannabinoid receptors, CB₁ and CB₂ receptors, in the brain [8]. These receptors are expressed in different areas of the brain, including PVN, amygdala, cerebellum, hippocampus, and cortex [1;2;13;16;22]. CB₁ and CB₂ receptors couple to G_{i/o} class of G-proteins and to the extracellular kinase (ERK) signaling pathway [4;8]. In our previous studies, we have reported that the cannabinoid-induced upregulation of 5-HT_{2A} receptors in two neuronal cell models is mediated by CB₂ receptors and ERK1/2 activation as it is inhibited in cells treated with CB₂, but not CB₁, shRNA lentiviral particles and by ERK1/2 inhibitors [17–19].

Activity of 5-HT_{2A} receptors in either PFCx or hypothalamic paraventricular nucleus (PVN) has been associated with several physiological functions and neuropsychiatric disorders such as stress response, anxiety & depression and schizophrenia [6;12]. Although the clinical manifestations of the cannabinoid-induced upregulation of 5-HT_{2A} receptors are currently under discussion, it has been suggested that repeated exposure to nonselective cannabinoid agonists might fasten the onset of the neuropsychiatric disorders described above [6;23;26;27;34]. Of note, recent preclinical studies indicated that chronic, but not acute, exposure to non-selective [30;31] or selective CB₂ receptor agonists induced anxiety-like behaviors in rodents [21].

Here, we investigated the effect of repeated exposure to CP55940 on the activity of 5-HT_{2A} receptors in the hypothalamic PVN. CP55940 is a nonselective cannabinoid agonist (CB₁ and CB₂ agonist, K_i: 0.58 nM and 0.68 nM for CB₁ and CB₂ receptors, respectively) [35]. We measured [(–)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl] (–)DOI, 5-HT_{2A/AC} agonist, induced changes in neuroendocrine responses, corticosterone and prolactin plasma levels, as an index of activity of 5-HT_{2A} receptors in PVN. We have previously demonstrated that the neuroendocrine effects of (–)DOI are mediated exclusively by activation of 5-HT_{2A} receptors, but not 5-HT_{2C} receptors, in rat PVN [39;41]. Here we reported a cannabinoid agonist-induced upregulation of 5-HT_{2A} receptors in hypothalamic PVN and increased-anxiety like responses in rats treated with CP55940. We hypothesize that these studies will further our understanding of the neurobiological mechanisms associated with repeated cannabinoid exposure.

2. MATERIALS AND METHODS

2.1 Drugs

CP55940, a CB₁/CB₂ agonist, was purchased from Tocris (Ellisville, MO). A fresh CP55940 solution (0.05 mg/ml) was prepared in Tween-80/ethanol/saline (1:1:18) prior to each dosing. (–) DOI [(–)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl] was purchased from Sigma-Aldrich Inc. (St. Louis, MO) and dissolved in 0.9% saline at one concentration (0.35 mg/kg, s.c.). All solutions were made fresh before administration and injected at a volume of 1 ml/kg.

2.2 Animal Experimental Protocol

Male Sprague-Dawley rats (225–275 g) were purchased from Harlan (Indianapolis, IN). The rats were housed two per cage in a temperature-, humidity-, and light-controlled room (12 hrs light/dark cycle, lights on 7:00 AM–19:00 PM). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC).

After arrival, the rats were allowed to acclimate to their environment for at least 7 days prior to the start of the treatment period. Eight rats were randomly assigned to each group. Cagemates were assigned to the same treatment group. Rats were injected with either vehicle (1ml/kg, i.p.) or CP55940 (0.05 mg/kg, i.p.) once a day for 7 days. Rats were sacrificed by decapitation 48 hrs after the last CP55940 injection. The rats were challenged with either saline (1ml/kg) or (-)DOI (0.35 mg/kg) 30 min prior to sacrifice. Trunk blood was collected for hormone assays and brain tissues were rapidly obtained and frozen in dry ice.

2.3 Radioimmunoassay

Plasma prolactin and corticosterone concentrations were determined by radioimmunoassays as previously described [7].

2.4 Western blots

Membrane-associated proteins were isolated using the ProteoExtract™ Native Membrane Protein Extraction kit (Calbiochem, La Jolla, CA) according to manufacturer's instructions. Briefly, PVN tissue was homogenized in extraction buffer I containing protease inhibitor cocktail. Homogenates were incubated for 15 mins at 4°C under gentle agitation, centrifuged 16,000 × g for 15 mins at 4°C, and then the supernatant containing the cytosolic fraction was collected. The pellet was incubated with extraction buffer II with protease inhibitor cocktail for 30 mins at 4°C under gentle agitation and centrifuged 16,000 × g for 15 mins at 4°C to isolate the membrane fraction contained in the supernatant. Expression of membrane-associated 5-HT_{2A} receptors in PVN was determined by Western blot as previously described [7]. Films were analyzed densitometrically using Scion Image software (Scion Corporation, Frederick, MD, USA) as previously described [7]. Each sample was measured on three independent gels. All samples were standardized to controls and normalized to their respective actin levels.

2.5 Quantitative Real-Time PCR

These reactions were prepared using QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA), the ABI 7500 fast real time PCR system (Applied Biosystems, Foster City, CA) and then data was analyzed using the comparative cycle threshold (Ct) method as described [36]. The primers used in this manuscript were: 5-HT_{2A} (F:5'-AACGGTCCATCCACAGAG-3', R:5'-AACAGGAAGAACACGATGC-3'), Gα_q (F:5'-AGTTCGAGTCCCCACCACAG-3', R:5'-CCTCCTACATCGACCATTCTGAA-3'), and GAPDH (F:5'-TGGAGTCTACTGGCGTCTTCAC-3', R:5'-GGCATGGACTGTGGTCATGA-3'). These primers have been previously validated [3;25;29].

2.6 Behavioral Tests

We used a separate group of rats to measure anxiety-like behaviors in rats injected with either vehicle (1ml/kg, i.p.) or CP55940 (0.05 mg/kg, i.p.) once a day for 7 days. 48 hrs after the last CP55940 injection, anxiety-like behaviors and locomotor activity were assessed in an elevated plus maze (Med Associates, St. Albans, VE) as previously described [32;37].

2.7 Statistics

All data are expressed as the mean ± S.E.M., where *n* indicates the number of rats per group. Data was analyzed by an unpaired Student's *t*-test or ANOVA (Newman-Keuls post-hoc test). GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD, USA) was used for statistical analyses.

3. RESULTS

We first examined the effect of repeated administration of CP55940, a CB₁/CB₂ receptor agonist [4], on the activity and expression of 5-HT_{2A} receptors in PVN. Rats were treated with CP55940 once a day for 7 days and then were challenged with (-)DOI (5-HT_{2A/2C} receptor agonist) 30 mins prior to sacrifice. We used this challenge to measure activity of 5-HT_{2A} receptors in PVN because the neuroendocrine effects of (-)DOI, are mediated exclusively by activation of 5-HT_{2A} receptors, but not 5-HT_{2C} receptors, in PVN [7]. In (-)DOI-challenged rats, we found significant ($p < 0.05$) increases in 5-HT_{2A} receptor-mediated prolactin (Fig.1A) and corticosterone (Fig.1B) plasma levels. The levels of prolactin increased from 28.1 ± 0.9 pg/ml in control rats challenged with (-)DOI to 44.60 ± 2.2 in CP55940-treated rats challenged with 0.35nM (-)DOI (Fig.1A). Plasma corticosterone levels increased from 21.5 ± 0.6 in control rats to 35.7 ± 0.7 in CP55940-treated rats (Fig.1B).

Upregulation of 5-HT_{2A} receptors could mediate this cannabinoid-induced phenomenon in hypothalamic tissue. Interestingly, we found a significant ($p < 0.01$) increase in the membrane-associated 5-HT_{2A} receptor protein levels in PVN of rats treated with CP55940 compared to controls (approx. 5-fold increase in PVN, Fig.1C). Increased mRNA synthesis, decreased receptor degradation, and/or increased translocation of these receptors from the cytosol to the membrane are the plausible mechanisms that could underlie the increased membrane-associated levels of 5-HT_{2A} receptors shown in Fig.1C. We then measured 5-HT_{2A} mRNA levels in PVN samples of either controls or CP55940-treated rats. In PVN, we did not find any significant CP55940-mediated changes in 5-HT_{2A} mRNA levels (Fig.1D) nor did we detect any modification in the levels of 5-HT_{2A} receptor-associated G-protein, G α_q (Fig.1D).

Repeated cannabinoid administration has been linked to increased anxiety, depressed mood, irritability and restlessness [5;10]. Here we want to determine whether the increased cannabinoid-induced activity of hypothalamic 5-HT_{2A} receptors is associated with anxiety-like behaviors in rats. We used a separate group of rats to measure anxiety-like behaviors in CP55940 treated animals 48 hours after the last cannabinoid administration. In the elevated plus maze, anxiety-related behavior is measured by the degree to which the rodent avoids the open (unenclosed) arms of the maze [32]. Since locomotor activity affects the interpretation of the elevated plus maze observations, we measured the number of transitions between the different arms of the maze as an index of locomotor activity as previously described [21].

We detected significant ($p < 0.05$) differences between vehicle and CP55940 treated rats when we measured the percent time spent in open arms of the plus maze (% open arms/total time, Fig.2A). Indeed, we detected that vehicle treated rats spent significantly ($p < 0.05$) more time in the open arms of the maze compared to CP55940-treated rats ($28.6 \pm 3.2\%$ and $16.4 \pm 4.8\%$ for vehicle and CP55940-treated rats, respectively). Interestingly, we did not detect any significant changes in the number of transitions between the different arms of the maze in vehicle or CP55940-treated rats (31.4 ± 2.9 and 33.0 ± 3.8 for vehicle and CP55940-treated rats, respectively) (Fig.2B).

4. DISCUSSION

Our results identified a cannabinoid agonist-induced increase of 5-HT_{2A} signaling in the hypothalamic PVN cells that was manifested by increased prolactin and corticosterone neuroendocrine responses to (-)DOI, a 5-HT_{2A/2C} receptor agonist (Fig.1A & 1B). We have demonstrated that the neuroendocrine effects of (-)DOI on the secretion of these two

stress hormones, are mediated by activation of 5-HT_{2A} receptors in PVN [39;41]. We used (-)DOI (0.35 mg/kg), a submaximal dose (close to EC₅₀) in these studies, since higher doses of (-)DOI may reach a ceiling effect that may prevent us from quantifying the cannabinoid-induced increases in 5-HT_{2A} receptor activity in this brain area, as previously reported [24]. The upregulation of 5-HT_{2A} receptors is a mechanism that could mediate the increased activity of the 5-HT_{2A} receptor. Indeed, we found increased membrane-associated 5-HT_{2A} receptor protein levels in PVN of CP55940-treated rats that were not associated with changes in 5-HT_{2A} mRNA (Fig.1C & 1D). Since 5-HT_{2A} receptors seem to be located mostly in the cytosol of neuronal cells [14;40], it is possible that repeated cannabinoid exposure may enhance the translocation of the receptor from the cytosol to the membrane and/or decrease the degradation of this receptor. Interestingly, we have reported that repeated exposure to cannabinoids is also associated with increased activity and protein expression of membrane-associated 5-HT_{2A} receptors in rat PFCx [17;18]. However, this latter phenomenon was associated with increased 5-HT_{2A} mRNA levels in rat PFCx suggesting that the mechanism by which CP55940 increase the protein expression and activity of 5-HT_{2A} receptor in brain might be brain-region specific.

Enhanced activity of 5-HT_{2A} receptors in hypothalamic PVN has been associated with several mood disorders including anxiety [6;15]. Repeated exposure to cannabinoids has been linked to increased anxiety, depressed mood, irritability, and restlessness [5;10]. When we used a separate group of rats to measure the effect of CP55940 on anxiety-like behaviors, we found that the time CP55940-treated rats spent in the open arms of the elevated plus maze was reduced compared to vehicle controls. This was not associated with changes in the number of transitions between vehicle and cannabinoid-treated rats, suggesting that repeated exposure to CP55940 enhances anxiety-like behaviors in rats without modifying locomotor activity. These studies are in line with preclinical reports that show chronic exposure to CP55940 and JWH133 (selective CB₂ agonist) induce anxiety-like behaviors in young adult [21] and adolescent rodents [31], respectively. Noteworthy, clinical studies have reported that chronic cannabinoid users have higher levels of anxiety compared with control groups [33]. Furthermore, the severity of anxiety symptoms increased as the level of cannabinoid use increased [9;28].

Further studies are required to identify the cannabinoid receptor and the specific mechanism by which CP55940 upregulates 5-HT_{2A} receptors in hypothalamic PVN. Our studies in two neuronal cell models suggest that CB₂, but not CB₁, receptors mediate this phenomenon [17–20]. Importantly, a 2011 report stated that Marijuana use among U.S. high school students has increased since 2008 [38]. The percentage of high school students who reported using marijuana or synthetic cannabinoids increased from 32% in 2008 to 39% in 2010 [38]. Despite this high incidence, the mechanism by which cannabinoid exposure produce some of its adverse effect is unknown. The significance of our studies is that they may be clinically relevant with respect to facilitating a better understanding of some mechanisms that may underlie the etiology of neuropsychiatric disorders in individuals repeatedly exposed to cannabinoids.

5. Conclusions

This study provides new insight into the cannabinoid agonist regulation of 5-HT_{2A} receptors in rat hypothalamic PVN. Furthermore, to the best of our knowledge this study is the first to show that repeated CP55940 treatment enhances 5-HT_{2A} receptor mediated increases in the neuroendocrine responses of two stress hormones, prolactin and corticosterone. We also detected increases in 5-HT_{2A} activity and protein expression in PVN and increased anxiety-like behavior in CP55940 treated rats. Therefore, this cannabinoid-induced upregulation of

5-HT_{2A} receptors might represent a potential adverse effect of long-term exposure to certain cannabinoid agonists.

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Abbreviations

5-HT	5-hydroxytryptamine
5-HT_{2A}	Serotonin 2A
Δ⁹-THC	Δ ⁹ -Tetrahydrocannabinol
ERK	extracellular kinase
PVN	hypothalamic paraventricular nucleus
CP 55,940	(-)- <i>cis</i> -3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]- <i>trans</i> -4-(3-hydroxypropyl)cyclohexanol (-)-DOI [(-)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl]

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Highlights

- Repeated CP55940 treatment enhances 5-HT_{2A}-mediated neuroendocrine responses.
- 5-HT_{2A} protein levels in PVN were increased after repeated CP55940 treatment.
- 5-HT_{2A} and Gαq mRNA levels in PVN were not modified by CP55940 treatment.
- CP55940 treatment increases anxiety-like behavior in rats.

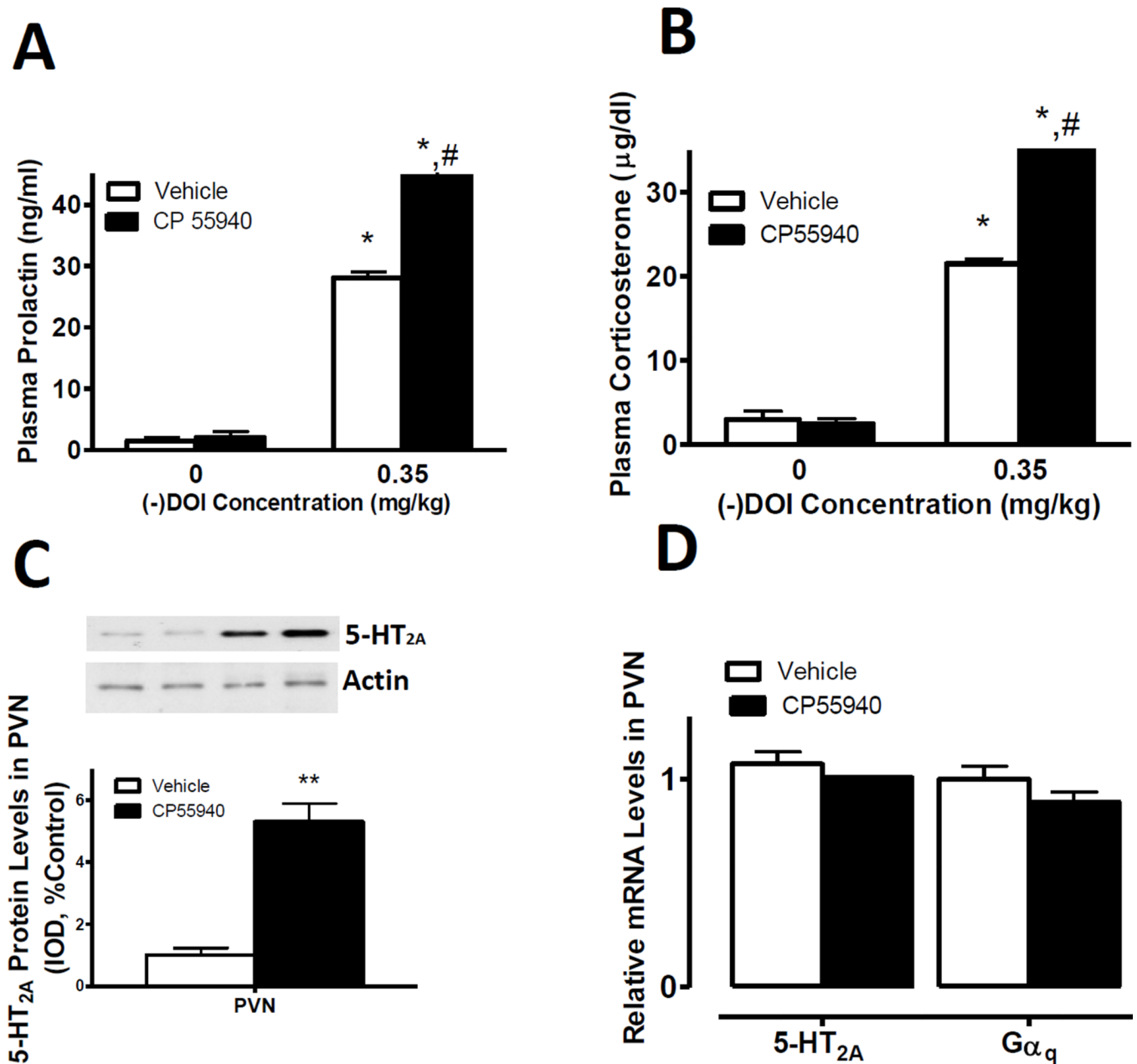


Figure 1. CP55940-induced enhanced activity and upregulation of 5-HT_{2A} receptors in hypothalamic PVN

(A and B) Increased 5-HT_{2A} receptor-mediated prolactin and corticosterone neuroendocrine responses in CP55940 and (-)DOI treated rats compared to vehicle and (-)DOI treated controls. * $p < 0.05$, significant effect of (-)DOI challenge in vehicle or CP55940 treated rats compared to vehicle-treated controls. # $p < 0.05$, significant effect (-)DOI challenge in CP55940 treated rats compared to (-)DOI challenge in vehicle-treated rats. (C) Increased membrane-associated 5-HT_{2A} receptor protein levels in the PVN of rats treated with CP55940 compared to vehicle treated controls. β -actin was used as a loading control and similar results were obtained in three separate experiments. (D) No significant changes in 5-HT_{2A} or G α_q mRNA levels in PVN of CP55940 treated rats compared to vehicle treated controls. Data represent the mean \pm SEM of 8 rats per group and were analyzed by one-way

ANOVA or t-Student test. * $p < 0.05$, significant effect of CP55940 compared with their respective vehicle-treated controls.

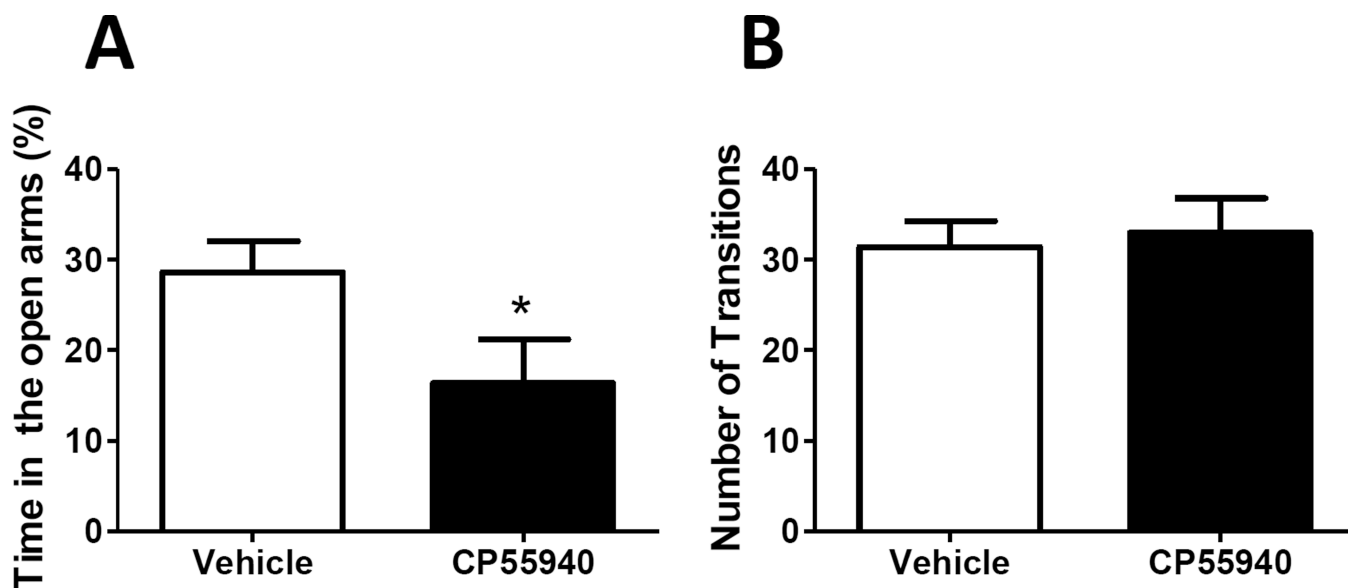


Figure 2. Effect of repeated CP55940 treatment on anxiety-like behavior in the elevated plus maze test

(A) Percent time spent in the open arms of the elevated plus maze was reported as a measure of anxiety-like behavior which was evaluated for a period of 5 minutes. (B) Transitions between the different arms of the maze was used as an index of locomotor activity. Mean \pm SEM, $n=6-8$ rats. * $p<0.05$, significant behavioral effect of CP55940 compared with their respective vehicle-treated control rats.