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Upregulation of CB_1 receptor binding in the ventromedial prefrontal cortex promotes proactive stress-coping strategies following chronic stress exposure

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Abstract

Accumulating evidence has revealed that dysregulation of the endocannabinoid system could contribute to the development of major depression. Studies carried out post-mortem in depressed suicide victims have revealed increased CB_1 receptor binding site density in the prefrontal cortex (PFC). Accordingly, exposure of rodents to chronic unpredictable stress (CUS) results in phenotypic changes that mirror those of human depression, including increased CB_1 receptor binding site density in the PFC. Our goal in these studies was to examine the effects of CUS on the density of CB_1 receptor binding sites in the rodent medial PFC and to explore the role of this alteration in the behavioral changes invoked by CUS. Rodents exposed to CUS exhibited increased CB_1 receptor maximal binding site density (B_{max}) within the ventromedial PFC, but not the dorsomedial PFC. To determine whether this change in the ventromedial PFC is an adaptive response, or alternatively, a consequence of chronic stress that contributes to the adoption of passive coping, we examined whether local CB_1 receptor blockade within the ventromedial PFC following CUS would significantly alter behaviors in the forced swim test (FST). CUS exposure significantly increased passive coping in the FST, and this was further augmented by discrete ventromedial PFC microinfusions of the CB_1 receptor antagonist AM251 prior to swim stress. Moreover, local CB_1 receptor blockade reduced active coping responses in CUS-exposed rats. These findings suggest that the increase in CB_1 receptor B_{max} observed in the ventromedial PFC

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of rodents exposed to CUS maintains proactive coping strategies following chronic stress exposure.

Keywords

CB_1 receptor; ventromedial prefrontal cortex; chronic unpredictable stress; forced swim test; microinfusion

The endocannabinoid system has recently emerged as a key component in the etiology of stress-related illnesses and could represent a novel therapeutic candidate for its treatment [1]. This system is comprised of a presynaptically located receptor (CB_1) and two endogenous ligands, *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), which are synthesized on-demand and serve to modulate excitatory, inhibitory, and monoaminergic neurotransmission in brain regions involved in the regulation of emotionality and stress [2,3]. Preclinical studies employing genetic deletion or chronic pharmacological antagonism of the CB_1 receptor reveal a behavioral and neuroendocrine profile that resembles the phenotype of major depression in humans [4]. Likewise, rats exposed to chronic unpredictable stress (CUS), an animal model of depression [5,6], exhibit reduced CB_1 receptor binding in subcortical limbic structures such as the hippocampus, hypothalamus, and ventral striatum [7-9].

While exposure to chronic stress and the development of a depressive phenotype are associated with reductions in endocannabinoid signaling in most brain regions, a different pattern has emerged in the prefrontal cortex (PFC). For example, CUS exposure induces a robust increase in CB_1 receptor mRNA and binding in the PFC [8,10,11]. Furthermore, this increase in CB_1 receptor binding in the PFC is normalized following chronic treatment with the monoamine uptake inhibitor imipramine [8] or URB597, an inhibitor of AEA degradation [11]. Similarly, increased CB_1 receptor density and signaling in the PFC has been reported in another animal model of depression, olfactory bulbectomy (OBX) [12]. These changes were linked to alterations in anxiety-like behavior in the open field test; and both the increase in CB_1 receptor density and increased anxiety behavior were reversed following chronic fluoxetine treatment [12].

Although these preclinical findings are intriguing, theses studies have only examined CUS- or OBX-induced changes in CB_1 receptor activity using whole PFC tissue samples. Thus, little is known regarding the precise anatomical subregion of the PFC affected by CUS exposure. Thus, we first sought to determine the effect of 21-day CUS exposure on CB_1 receptor binding parameters specifically within the ventromedial PFC. This prefrontal subregion was chosen because of its structural and functional homology to the subcallosal cingulate gyrus (SCG) in humans, a cortical area that shows abnormal metabolic activity in major depression and is a target for deep brain stimulation in drug refractory depressives [13]. We also examined the dorsomedial PFC as a control region to determine the specificity of changes in the PFC evoked by CUS.

These preclinical data are in agreement with post-mortem reports from depressed suicide victims demonstrating that CB_1 receptor protein expression, binding site density, and signal transduction are all increased in the PFC of these individuals [14-16]. Collectively, this suggests that increased CB_1 receptor binding activity within the PFC is associated with the development of major depression. However, it is not currently known whether this increase in prefrontal CB_1 receptor binding represents a compensatory adaptive response initiated to dampen the behavioral symptoms of depression induced by chronic stress, or alternatively, a driving factor that contributes to the development of these changes. Thus, the second goal of

this study was to explore the contribution of CUS-induced alterations in CB_1 receptor binding within the ventromedial PFC with respect to coping strategies in the forced swim test (FST).

Seventy-day-old male Sprague-Dawley rats (300 g; Charles River, Montreal, Canada) were housed in groups of three in standard maternity bins lined with contact bedding. Colony rooms were maintained at 21 °C on a 12-hr reverse light/dark cycle (lights off at 0700hr). All rats were given ad libitum access to Purina Rat Chow and tap water. The guidelines of the Canadian Council on Animal Care were followed and the experiment was approved by the Animal Care Committee of the University of British Columbia.

Rats were randomly assigned to either CUS or control (CON) conditions prior to beginning the study. The CUS paradigm employed consists of 2-3 stressors per day for 21 days from the following list: 1 hr tube restraint; 1 hr exposure to social crowding with white noise/ stroboscopic illumination; 5 min forced swim exposure; 18 hr food and/or water deprivation; 3 hr cage rotation to alter dominance hierarchies; and 18 hr social isolation in damp bedding. For studies where the FST was used as a behavioral endpoint, forced swim exposure in the CUS paradigm was replaced with 1 hr cage tilt at a 30° angle. All stressors were randomized and separated by a period of at least 2 hr. Rats assigned to the CON condition were handled three times per week for the duration of study.

On the morning after the final day of CUS, rats used for CB_1 receptor binding analyses were decapitated and their brains were rapidly removed (n=6/group). The dorsomedial PFC (consisting of anterior cingulate and motor cortices) and ventromedial PFC (consisting of the prelimbic and infralimbic cortices) were dissected, flash-frozen in liquid nitrogen, and stored at -80°C until analysis (see Fig. 1 for boundaries of dissection). CB_1 receptor radioligand binding was performed using a Multiscreen Filtration System with Durapore 1.2-μM filters (Millipore, Bedford, MA) as described previously [17]. Membranes (10 μg protein per incubate) were added to wells containing 0.1, 0.25, 0.5, 1.0 or 2.5 nM of [^{3}H] CP55940, a cannabinoid CB_1 receptor agonist. Ten μM Δ^9-tetrahydrocannabinol was used to determine non-specific binding. The maximal CB_1 receptor binding site density (B_{max}) and affinity of [^{3}H] CP55940 for the CB_1 receptor (K_D) were determined by nonlinear curve fitting to the single site binding equation using GraphPad Prism (San Diego, CA, USA).

Independent t-tests conducted between control and CUS-exposed animals revealed that within the ventromedial PFC, the maximal binding site density (B_{max}) of the CB_1 receptor was significantly greater in CUS-exposed animals [$t(9) = 3.85$, $p < .005$], with no significant differences in K_D. However, in the dorsomedial PFC, control and CUS-exposed animals did not differ significantly with respect to B_{max} or K_D. These results suggest that the maximal binding site density of CB_1 receptors is significantly higher within the ventromedial PFC of CUS-exposed animals (Fig. 2).

We hypothesized that the increase in ventromedial prefrontocortical CB_1 receptor binding counteracts the effects of CUS for the following reasons. First, local activation of CB_1 receptors or inhibition of AEA hydrolysis specifically within the ventromedial PFC has been shown to promote antidepressant-like responding in the FST [18,19]. Second, under neuropathological conditions, glial cells release an increased amount of endocannabinoids and over-express CB_1 receptors in the PFC, which has been argued to constitute an endogenous defense mechanism that prevents additional cell damage [20]. In agreement with this notion, CB_1 receptor knockout mice have been shown to exhibit dysregulation of the hypothalamic-pituitary-adrenal axis along with exacerbated excitotoxic/ neuroinflammatory responses in the PFC [21]. Given the negative impact of CUS exposure

and the neuroprotective capacity of CB_1 receptors in the PFC, it is likely that the increase in CB_1 receptor binding observed in the present study reflects a compensatory response.

To further examine the functional relevance of this change in CB_1 receptor binding, separate cohorts of animals were randomly assigned to one of four groups (n=7/group): 1) CUS-VEH; 2) CUS-AM251; 3) CON-VEH; 4) CON-AM251. These animals were implanted with bilateral cannula aimed at the ventromedial PFC prior to initiation of CUS, which occurred approximately 10-13 days post-surgery (see Fig. 1). Rats were anesthetized with a cocktail of 100 mg/kg of ketamine hydrochloride and 7 mg/kg xylazine and implanted with guide cannula using the coordinates AP = + 3.0; ML = +/- 0.7; DV = -3.4, as described previously [22]. Following behavioral testing, tissue was sliced and stained with cresyl violet, and cannula placements were verified according to the stereotaxic atlas of Paxinos and Watson [23].

On the subsequent 2 days following CUS exposure, rats were tested in the FST during the middle third of the animals' dark cycle. Glass cylindrical containers (diameter 35 cm, height 45 cm) were filled with 30 cm of water maintained at 24±1°C. Consistent with the modified method of testing in the FST, animals were subjected to two swim sessions [24]. The first swim session was a 15-min pre-exposure session, followed by a 5-min test session 24 hr later. During the test session, the duration of immobility, swimming, and struggling was videotaped and scored by trained assistants blinded to experimental conditions (see [25] for a description of scoring criteria for each behavioral component).

Rats received intra-ventromedial PFC infusions of the CB_1 receptor antagonist AM251 (Tocris Cookson Ltd., Bristol, UK) or vehicle 30 min prior to the day 2 swim session and were placed back into their home cages until testing began. AM251 (0.28 ng) or vehicle (1 part dimethyl sulfoxide, 9 parts 0.9% sterile saline) was administered at a volume of 0.2 μl/side directly into the ventromedial PFC as described previously [22]. This dose was chosen in accordance with recent studies demonstrating behavioral and neuroendocrine effects following intracranial microinjection of AM251 at this dose [22,26].

A two-way ANOVA revealed a significant effect of interaction between stress and drug treatment on immobility in the FST [$F(1,24) = 6.36$, $p < .02$]. Post-hoc analysis revealed that control animals receiving vehicle infusions (CON-VEH) spent significantly less time in an immobile posture compared to CUS-exposed rats receiving vehicle infusions (CUS-VEH; $p < .05$) and AM251 infusions (CUS-AM251; $p < .01$), but not relative to control rats receiving AM251 infusions (CON-AM251). Moreover, animals in the CUS-AM251 group showed significantly greater levels of immobility relative to those in the CUS-VEH group ($p < .05$; Fig. 3). A two-way ANOVA also revealed a significant effect of group on swimming behavior in the FST [$F(1,24) = 3.84$, $p < .05$]. Follow-up analyses showed that although animals in the CON-VEH and CUS-VEH groups did not differ significantly, those in the CUS-AM251 group did demonstrate a significant reduction in swimming compared to those in the CUS-VEH ($p < .05$), CON-VEH ($p < .05$), and CON-AM251 ($p < .05$) groups. There was no significant effect of group on struggling behavior (Fig. 3).

The results of this study support our hypothesis that increased ventromedial PFC CB_1 receptor density opposes the effects of CUS in the FST. Rats in both CUS-exposed groups (CUS-VEH and CUS-AM251) showed greater levels of immobility compared to control rats, which is reflective of enhanced passive stress coping strategies. Furthermore, rats in the CUS group pretreated with intra-ventromedial PFC infusions of the CB_1 receptor antagonist AM251 prior to swim stress displayed the highest levels of immobility, even significantly more than those in the CUS group receiving vehicle infusions. Those in the CUS-AM251 treatment group also spent significantly less time swimming compared to those in all other

treatment groups, which is suggestive of a reduced reliance on active, escape-directed coping strategies. Alterations in swimming behavior are thought to be mediated by changes in 5-HT transmission [24], and consistent with this, the antidepressant-like effect of intra-ventromedial PFC CB_1 receptor activation occurs via interactions with this system [18,19]. Thus, it is possible that the increase in CB_1 receptor binding observed in the present study may be acting to facilitate 5-HT output from midbrain monoaminergic nuclei. Together, these findings are consistent with our hypothesis that CB_1 receptor activation in the ventromedial PFC promotes active coping in CUS-exposed rats. Moreover, these data suggest that CUS increases passive coping strategies via a mechanism that likely does not involve an increase in endocannabinoid signaling, but that increased endocannabinoid signaling can mitigate against the effects of CUS. Although this may be the most parsimonious explanation, it is also possible that local CB_1 receptor antagonism exacerbated passive coping strategies in the FST by blocking stress-induced sensitization of a CB_1 signaling pathway that occurs either independently from receptor binding changes, or through discrete receptor populations that do not show changes in binding characteristics.

The CUS-induced increase in CB_1 receptor binding specifically within the ventromedial PFC is intriguing given that this region is a key determinant of depressive-like behavior and antidepressant responses both in clinical observations and preclinical studies. The rodent ventromedial PFC is functionally homologous to the SCG in humans [13,27], and notably, cellular and neuroimaging studies have revealed a reduction in immediate early gene expression and a substantial loss in gray matter in the SCG of depressed individuals [28,29]. Moreover, a variety of clinical interventions including pharmacological antidepressants, electroconvulsive shock treatment, and deep brain stimulation have all been associated with changes in activity in the SCG coinciding with symptom improvement [28]. Accordingly, stress-susceptible rodents exposed to chronic social defeat stress exhibit similar reductions in immediate early gene activity in the ventromedial PFC, while optogenetic stimulation of this population of neurons produces antidepressant-like effects in these animals [29]. Furthermore, deep brain stimulation of the rodent ventromedial PFC has also been shown to promote a robust increase in active coping responses in the FST that is dependent on the integrity of the serotonin (5-HT) system [30]. In agreement with this last report, local activation of CB_1 receptors within the ventromedial PFC has been shown to elicit a similar 5-HT-mediated response in the FST [18]. Given that activation of CB_1 receptors within the ventromedial PFC elicits active coping responses similar to optogenetic and deep brain stimulation, it is not surprising that local pharmacological CB_1 receptor blockade further exacerbated passive coping behaviors (immobility) and reduced escape-directed behaviors (swimming) in the FST. These data are consistent with the fact that CB_1 receptors in this region predominantly exist on GABAergic interneurons and the net effect of CB_1 receptor activation in the medial PFC is an increase in the activation and outflow of projection neurons [22,31], similar to what would occur following focal stimulation of this region. It is also possible, however, that the CUS-induced increase in CB_1 receptor binding is occurring primarily on astrocytes, where activation of CB_1 receptor signaling can potentiate excitatory synaptic transmission [32]. Future work will be required to determine the neuronal or glial population mediating this effect.

The precise neurobiological mechanisms that are driving this change in CB_1 binding are currently unknown. However, our laboratory has previously shown that a decrease in PFC AEA content also accompanies the increase in CB_1 receptor binding [8], and pharmacological inhibition of FAAH during CUS exposure prevents the increase in CB_1 receptor mRNA expression that occurs within the PFC [11]. Accordingly, exposure to chronic stress enhances FAAH activity in the medial PFC and amygdala [33], and moreover, this change in FAAH is necessary for the stress-induced changes in amygdalar structure and function [34]. Consequently, we speculate that the FAAH-mediated decrease in AEA

content indirectly stimulates this compensatory up-regulation of CB_1 receptor binding in the ventromedial PFC in an effort to maximize the diminishing AEA signaling pool induced by CUS exposure. Local pharmacological facilitation of AEA/CB_1 receptor signaling elicits antidepressant and anxiolytic responses in the FST and elevated plus maze, respectively [19,35], so in this regard it is possible that this response is a mechanism engaged to curb changes in stress coping and anxiety induced by chronic stress. However, it should be noted that previous reports have failed to show enhanced FAAH activity following exposure to CUS [8,11]. Thus, it is possible that the increase in CB_1 receptor binding could also be a result of diminished AEA biosynthesis, although this has yet to be empirically evaluated.

In conclusion, the findings from the present study support the notion that increased CB_1 receptor binding in the ventromedial PFC facilitates proactive stress coping responses in response to CUS exposure. Future studies are needed to determine whether the local reduction in prefrontal AEA content is driving the increase in CB_1 receptor binding, and whether pharmacologically maintaining AEA tone over the course of CUS exposure can prevent this increase in binding and produce a stress-resilient phenotype via interactions with the 5-HT system.

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Highlights

- CUS exposure increased CB_1 receptor binding sites in the rat ventromedial PFC
- Local ventromedial PFC CB_1 blockade exacerbated CUS-induced immobility in the FST
- Ventromedial PFC CB_1 blockade also decreased active coping in the FST following CUS
- CUS-induced upregulation of ventromedial PFC CB_1 binding serves an adaptive purpose

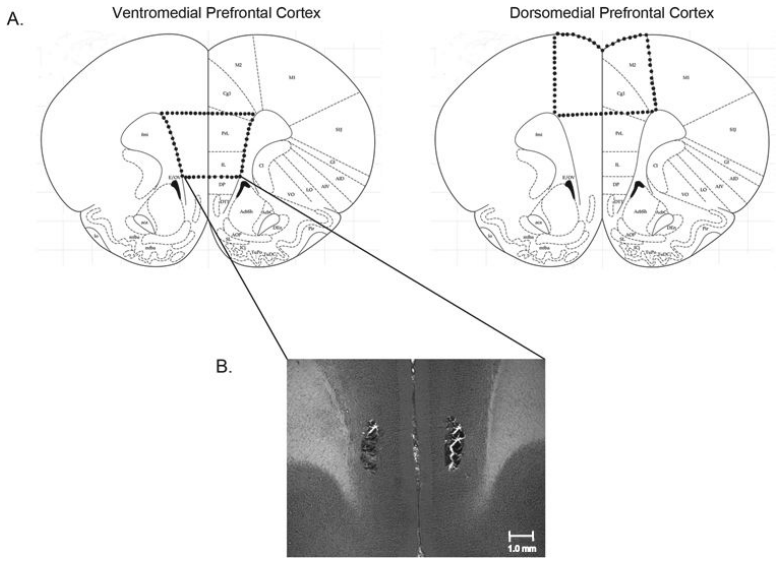

Figure 1.
Diagram depicting the boundaries for dissection for dorsomedial and ventromedial prefrontal cortex tissue extractions (above). Representative photomicrograph showing a bilateral cannula placement for rats receiving microinfusions into the ventromedial prefrontal cortex (below).

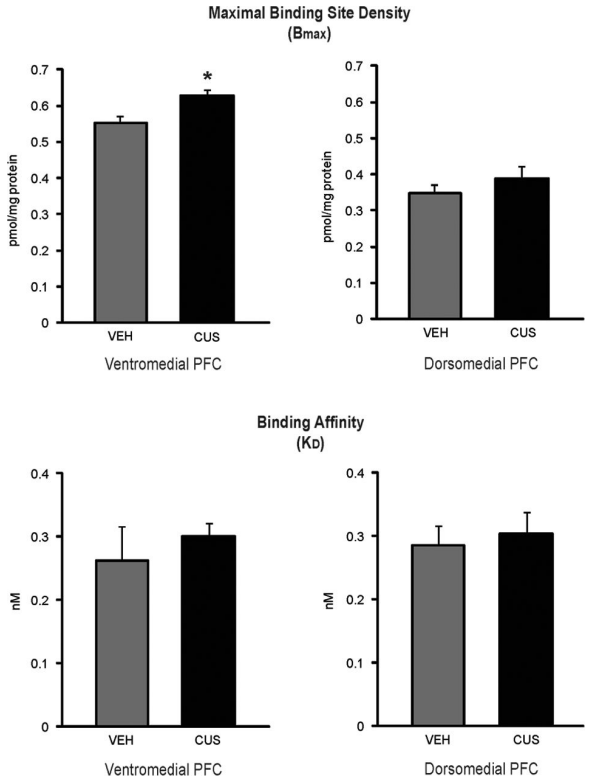

Figure 2.

The effect of chronic unpredictable stress (CUS) on the maximal binding site density (B_{max} in pmol/mg protein) and binding affinity (K_D in nM) of CB_1 receptors in the dorsomedial and ventromedial prefrontal cortex (n=6/group). * denotes $p < .05$.

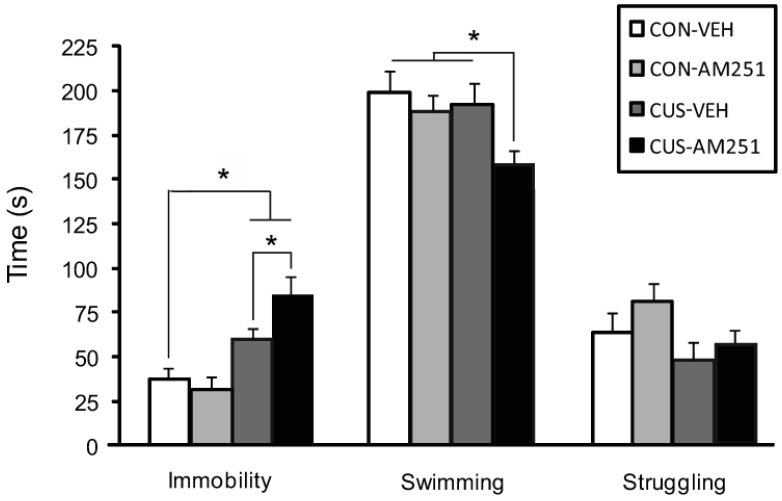

Figure 3.

The effect of local ventromedial PFC administration of the CB_1 receptor antagonist AM251 (0.28 ng) on immobility, swimming, and struggling behaviors in the forced swim test in control and CUS-exposed animals (n=7/group). * denotes $p < .05$.