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Cannabinoid receptor expression and phosphorylation are differentially regulated between male and female cerebellum and brain stem after repeated stress: Implication for PTSD and drug abuse

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ABSTRACT

Recent study demonstrated a close relationship between cerebellum atrophy and symptom severity of pediatric maltreatment-related posttraumatic stress disorder (PTSD). It has also been known that females are more vulnerable than males in developing anxiety disorders after exposure to traumatic stress. The mechanisms are unknown. Because cannabinoid receptors (CB_1 and CB_2) are neuroprotective and highly expressed in the cerebellum, we investigated cerebellar CB expression in stressed rats. Young male and female Sprague-Dawley rats were given 40 unpredictable electric tail-shocks for 2 h daily on 3 consecutive days. CB_1 and CB_2 mRNA and protein levels in rat cerebellum and brain stem were determined using quantitative real-time PCR and Western blot, respectively. Two-way ANOVA revealed significant gender and stress effects on cerebellar CB_1 mRNA expression, with females and non-stressed rats exhibiting higher CB_1 mRNA levels than the males (3 fold, $p < 0.01$) and stressed rats (30%, $p < 0.01$), respectively. CB_1 and CB_2 mRNA levels in brain stem were also greater in female rats than males ($p < 0.01$, $p < 0.05$, respectively). Repeated stress increased the level of phosphorylated CB_1 receptors, the inactivated CB_1, in rat cerebellum ($p < 0.01$), particularly in female rats as revealed by the significant gender × stress interaction. Thus, repeated severe stress caused greater CB_1 mRNA suppression and CB_1 receptor phosphorylation in female cerebellum that could lead to increased susceptibility to stress-related anxiety disorders including PTSD.

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Mounting evidence implicates a critical role of cannabinoid receptors (CB_1 and CB_2) in stress-related anxiety disorders and emotional memory. CB_1 knockout animals show hypersensitivity to stressful stimuli, decreased sociability, increased aggressive and anxiety-like behaviors and disrupted response to anxiolytic drugs [19,28,46]. CB_1 knockout animals and CB_1 antagonist-treated animals also show marked inability to extinguish fear memory [27]. These characteristics parallel the core clinical features of individuals with post-traumatic stress disorder (PTSD), i.e. reduced threshold of fear, impaired extinction of fear memory, vivid recall or flashbacks of traumatic memories and a high prevalence of cannabis abuse [23,36,49].

Despite the highest expression of cannabinoid receptors (CB_1 and CB_2) found in the cerebellum [20,25,48], its functional role remains to be established. To our knowledge, no one has reported cerebellar cannabinoid receptor expression under stressed conditions.

It is known that females are more vulnerable to traumatic stress and show a higher prevalence of stress-related anxiety disorders than males (2–3 fold) [7,39]. Yet many animal model studies of stress are based on male animals only; the generalizability of the male animal-based findings for females is limited accordingly. In this study, we examined CB_1 and CB_2 expression levels in the cerebellum and brain stem of young adult male and female rats, and the responses to repeated tail-shock stress.

Young juvenile male and female Sprague-Dawley rats (Taconic Farms, Germantown, NY, USA) weighing 100–150 g (5–6 weeks old) were unisex and pair-housed, with wood chip bedding, and maintained at a room temperature of $22 \pm 2\,^{\circ}C$ on a 12-h light-dark schedule (lights on 1800 h), and left undisturbed for 7 days before the start of the stress protocol that consisted of one 2-h per day session of restraint immobilization stress plus unpredictable repeated tail-shocks over 3 consecutive days. The animals were restrained in a Plexiglas tube and given 40 electric shocks (2 mA, 3 s duration) at varying intervals (140–180 s). Animals in the control group were handled daily as those in the stress group, but without going through the stress procedure.

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After the last stress session on day 3, both the control animals and the stressed animals were decapitated after light anesthesia with halothane. The brains were rapidly removed, dissected and kept at −70 °C for real-time PCR and western blotting (Appendix 1). Half of the dissected cerebellum and brain stem of each animal were homogenized for total RNA extraction with RNeasy kit (Qiagen) and reverse transcription into first-strand cDNA with a reverse transcriptase kit (Sigma, St. Louis).

The qPCR and western blot data were represented as mean ± s.d. The effects of gender and repeated stress on CB_1 and CB_2 receptor expression and phosphorylation in cerebellum and brain stem were analyzed using two-way ANOVA. As significant gender × stress interactions were found in CB_1 mRNA expression and receptor phosphorylation, one-way ANOVA was conducted to evaluate the effect of stress within each sex. $p < 0.05$ was considered statistically significant.

The qPCR data showed that CB_1 mRNA levels were much higher than CB_2 mRNA in the cerebellum and brain stem of both male and female control rats as shown by the CB_1:CB_2 mRNA ratio (>30 fold) based on the Ct values of each receptor (Appendix 2). This is consistent with previous findings of high CB_1 expression in cerebellum [20].

Two-way ANOVA revealed significant main effects of gender ($p < 0.01$) and repeated stress ($p < 0.01$), as well as significant gender × stress interaction ($p < 0.01$) on CB_1 mRNA expression in rat cerebellum, with females and non-stressed rats exhibited higher CB_1 mRNA levels than the males (3 fold) and stressed rats (30%), respectively (Fig. 1A). The significant gender × stress interaction suggests a greater stress-induced reduction of CB_1 mRNA in the female cerebellum ($p < 0.01$) than in males ($p < 0.05$) as revealed by the within-sex 1-way ANOVA (Fig. 1A). Two-way ANOVA showed significant gender, but no stress effect on CB_1 mRNA expression level in the brain stem, with female rats showing higher level than male rats ($p < 0.01$) (Fig. 1B). CB_2 mRNA expression level tended to be greater in the cerebellum and significantly greater in the brain stem ($p < 0.05$) of female rats than males (Fig. 1C and D). No significant stress effect was found in brain stem CB_2 mRNA.

No significant gender and stress effects were found on total CB_1 and CB_2 proteins expressed in rat cerebellum and brain stem (Figs. 2 and 3). However, phosphorylated CB_1 receptor (p-CB_1), the inactivate form of CB_1 receptors, was significantly increased in the cerebellum of the stressed rats (2-way ANOVA, $p < 0.01$), especially in the stressed females (Fig. 2A). Further, a significant stress × gender interaction existed in cerebellar p-CB_1 protein level suggesting a greater p-CB_1 level in the female cerebellum as supported by the within-sex 1-way ANOVA ($p < 0.01$) (Fig. 2A). Because CB_1 receptor phosphorylation has been shown to be associated with increased internalization and desensitization of CB_1 receptors [18], the increased p-CB_1 receptors suggest increased inhibition of CB_1 receptor activity in the cerebellum of stressed animals.

The gender difference in cerebellar CB_1 and CB_2 mRNA expression is consistent with the reports of greater endocannabinoid content in the brains of female rats [5] and a greater CB_1 mRNA expression in the blood cells of female human subjects [34]. Gender differences have also been reported in the amount of self-administered CB_1 agonists, in brain metabolism and behavioral effects of δ^9-tetrahydrocannabinol (δ^9-THC, the main active component of marijuana and potent CB_1 agonist) in rodents, with females self-administering more CB_1 agonists, producing significantly more of the active metabolite of THC and enhanced THC-induced behavioral effects including antinociception, motoric effects, catalepsy and hypothermia [10,16,33,44,45]. Although the mechanism is unknown, sex hormones and the estrous cycle may be involved in the gender difference in CB_1 and CB_2 receptor expression. There are reports that ovariectomy (OVX) decreased, whereas acute and chronic estradiol or progesterone administration increased the density of cannabinoid receptors in the limbic forebrain and in the hypothalamic nucleus of intact and OVX female rats [40]. As the present female rats were in the pubertal stage and their estrous cycles were not controlled, this could have potentially contributed to the higher variability in CB_1 and CB_2 receptor expression in the female brain.

The higher CB_1 mRNA expression levels but lower CB_1 protein expression levels in the brain stem of females than males suggest a differential regulation of CB_1 mRNA and protein expression between the sexes. While the mechanism is unknown, a potentially greater turnover/degradation rate of CB_1 receptor protein in the females could have triggered a need for greater CB_1 mRNA expression. Differential modification of the CB receptor (phosphorylation, glycosylation) induced by sex hormones could cause sex-dependent changes in the affinity and immunogenic activity of CB receptors as detected by the antibodies in this study and others [14]. Furthermore, the greater metabolic rate of cannabinoids reported in the females could cause a parallel increase in CB_1 protein turnover and CB_1 mRNA expression.

A difference in circulating corticosterone level could also be responsible for the observed sex-dimorphism in CB_1 mRNA expression. It is known that females have higher basal corticosteroid levels and greater stress-induced corticosteroid secretion but slower corticosteroid clearance from circulation [8,9]. Chronic exposure to corticosterone, CB_1 agonists and cannabinoids has been shown to downregulate CB_1 receptor density, CB_1 receptor binding and CB_1 mRNA expression in brain regions such as cerebellum and hippocampus [15,22,26,37,41].

Although no significant stress effect was found in CB expression in brain stem, this may reflect the technical limitations of present study. Because brain stem tissue homogenates were used in this study, the results are not conclusive regarding changes in CB receptor expression in specific nuclei of the brain stem. Further immunohistochemistry/*in situ* hybridization studies are required to determine specific changes in cannabinoid receptor expression in these nuclei after stress and their correlations with behavioral changes in PTSD models. Cannabinoid receptors expressed in the periaqueductal gray, locus coeruleus, raphe nuclei, reticular activating system, solitary tract, vestibular and cochlear nuclei of brain stem are thought to play important roles in the regulation of pain sensation, sleep-awake cycle, acoustic startle response, cardiovascular reactivity that are frequently altered in subjects with PTSD [1,2,12,30–32,38,48].

Since stress enhances norepinephrine (NE) synthesis/turnover in the LC and its subsequent release in the basolateral amygdala via alpha1A adrenergic receptor located in the presynaptic terminals, intense sympathetic activation and local NE release could regulate CB expression/activity via the release of eCBs. Alternatively, CB_1 receptors in the brain stem could potentially interacts with the alpha2-adrenergic autoreceptors to inhibit norepinephrine release from the adrenergic neurons of the locus coeruleus under physiological conditions [42]. Our studies show that the stress differentially suppressed brain adrenergic receptor mRNA expression between male and female rats, with a sex-dimorphism opposing that of brain CB mRNA expression (manuscript in preparation). Thus, brain CB and adrenergic systems could interact in central regulation of cardiovascular response in stress-induced anxiety disorders [6].

A recent study showed that acute maternal deprivation significantly increased neuronal apoptosis in the cerebellum of male rat pups and that induction was attenuated by inhibiting endogenous cannabinoid (eCBs) degradation [24]. As brain eCBs activity is mainly mediated by CB_1 receptors, that study implicates an important anti-apoptosis role of CB_1 receptor in stressed cerebellum. Another brain imaging study showed significant correlations between structural volumes of the cerebellar hemispheres and clin-

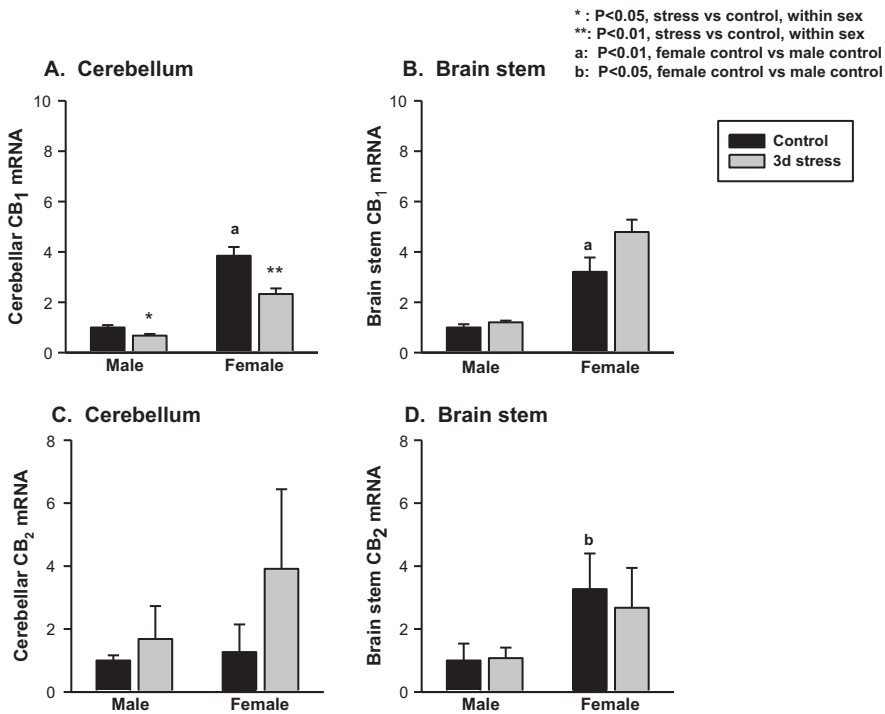

Fig. 1. Effects of gender and stress on CB_1 and CB_2 mRNA. (A), Cerebellar CB_1 mRNA level was significantly higher in the female rats ($n = 16$) than in the males ($n = 16$, $p < 0.01$) and in the non-stressed animals ($n = 16$) than in the stressed animals ($n = 16$, $p < 0.01$), respectively. Three days repeated stress down-regulated CB_1 mRNA level in male ($p < 0.05$) and female rats ($p < 0.01$) when compared with same sex controls; (B), Brain stem CB_1 mRNA was significantly greater in female rats than in male rats ($p < 0.01$) but no stress effect was found; (C), Cerebellar CB_2 mRNA was not affected by sex or stress; (D), Brain stem CB_2 mRNA level was significantly higher in female rats than in the males ($p < 0.05$). The mean value of the male control group (mean ± s.d.) was used as the arbitrary reference (=1). Black column: control rats; gray column, stressed rats, a, $p < 0.01$, male control vs. female control; b, $p < 0.05$, male control vs. female control; $^*p < 0.05$; $^{**}p < 0.01$, within-sex 1-way ANOVA.

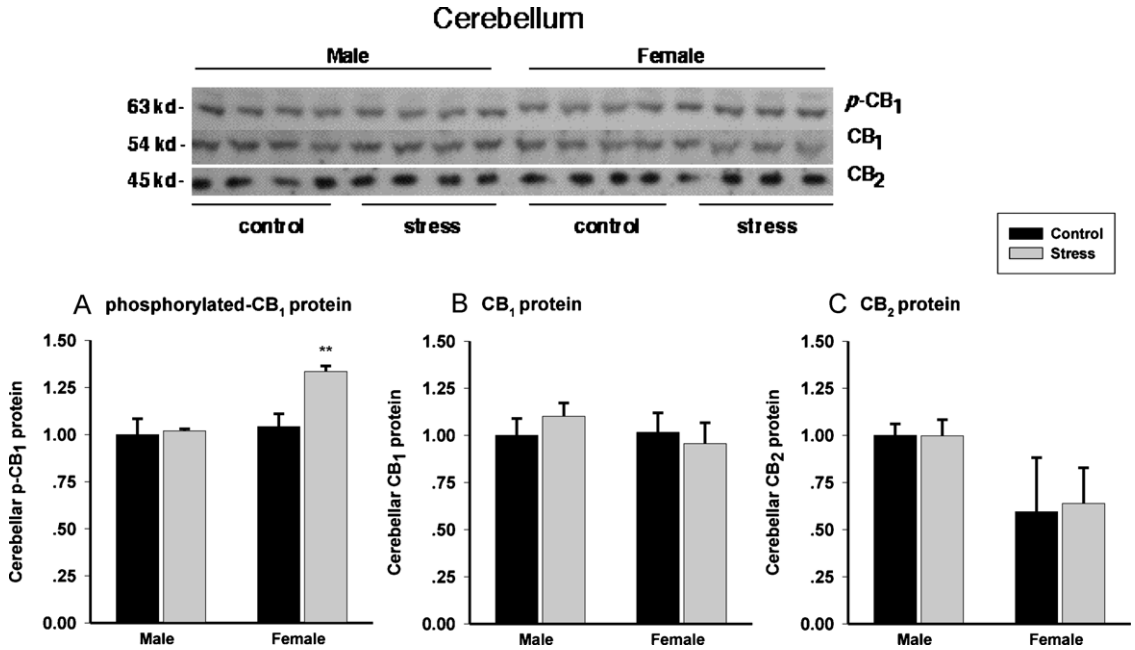

Fig. 2. Western blotting of CB proteins in rat cerebellum. *Top panel*, representatives of cerebellum western blotting: twenty micrograms (μg) of rat cerebellum tissue homogenates were resolved on SDS-PAGE gel and incubated with the specific CB antibodies and detected using an ECL western blot detecting system. *Bottom pane*, (A), phosphorylated CB_1 proteins (p-CB_1); (B), semi-quantitative western blotting of total CB_1 proteins; (C), CB_2 proteins and; (C) in rat cerebellar tissue homogenates with or without 3 days repeated tail-shock stress. The mean value (mean ± s.d.) of the male control group ($n = 8$) was used as the arbitrary reference (=1) for other groups ($n = 8$). Two-way ANOVA revealed significantly elevated p-CB_1 protein in the stressed animals, primarily in the females, $^*p < 0.05$; Black column, control group; gray column, stress group ($n = 8$ each group).

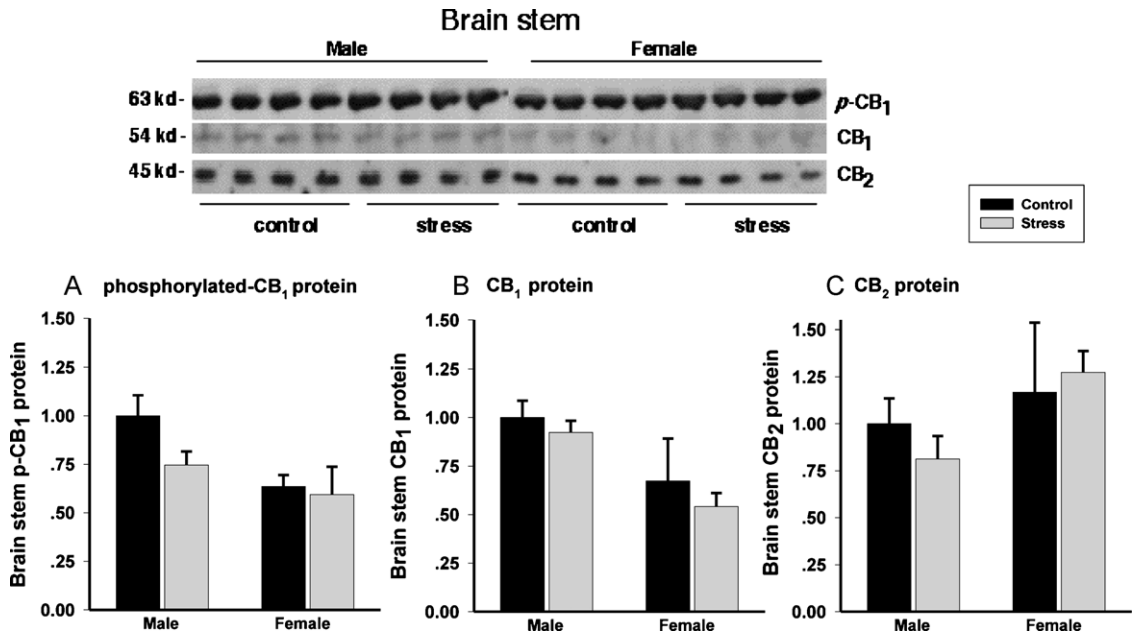

Fig. 3. Western blotting of CB proteins in rat brain stem. *Top panel*, representatives of cerebellum western blotting: twenty micrograms (μg) of rat brain stem tissue homogenates were resolved on SDS-PAGE gel and incubated with the specific CB primary antibodies and detected using an ECL western blot detecting system. *Bottom panel*, semi-quantitative western blotting of: (A) phosphorylated CB_1 proteins (p-CB_1); (B) total CB_1 proteins and; (C) CB_2 proteins in rat brain stem tissue homogenates with or without 3 days repeated tail-shock stress. The mean value (mean ± s.d.) of the male control group ($n = 8$) was used as the arbitrary reference (=1) for other groups ($n = 8$). Black column: control group; gray column, stressed group.

ical symptoms in pediatric maltreatment-related PTSD. The PTSD subjects had significantly reduced volume in both the left and the right hemispheres and in total cerebellum volume [11]. These studies implicate a link between stress-induced apoptosis and cerebellum atrophy in PTSD although the exact causative factors and the time course of the atrophy remain to be established. Our study suggests that chronic stress-induced CB_1 receptor suppression in early development could be involved in the cerebellar neuronal apoptosis or structural atrophy in PTSD.

It is known that females are more vulnerable than males after exposure to trauma [4,28]. The molecular mechanism is unknown. In this study, the stressed female rats showed a greater reduction in CB_1 mRNA expression but a greater increase in cerebellum CB_1 receptor phosphorylation (at serine 316) than the stressed males. Because phosphorylation at serine 316 of CB_1 receptor by protein kinase C is a key post-translational regulatory mechanism underlying rapid CB_1 receptor internalization and desensitization by disrupting cannabinoids-mediated activation of inwardly rectifying potassium current and depression of P/Q-type calcium channels x [18], this phosphorylation of CB_1 receptors would represent another novel mechanism of rapid CB_1 inhibition in the cerebellum of stressed female rats. Other studies suggest that it is the rate of change rather than absolute level that can bring about swings in mood and anxiety.

It is tempting to speculate that chronic stress-induced CB_1 receptor downregulation and inhibition could also be involved in increased marijuana use and cerebellum dysfunction such as altered time sense in PTSD [47]. While further studies are needed to evaluate this notion, impaired time sense has been reported in marijuana users who exhibited cerebellar hypoactivity in response to delta-9-tetrahydrocannabinol [29]. Because marijuana use is common among PTSD subjects and is correlated with the severity of PTSD symptoms [3], the greatly altered CB_1 mRNA expression and receptor phosphorylation/desensitization in stressed female cerebellum is consistent with the literature that women are more vulnerable to traumatic stress, become drug dependent more quickly and are more likely to experience craving than men [4,7,13,17,21,35,43].

In summary, we observed significant gender and repeated stress effects and their interaction on CB_1 mRNA expression and CB_1 receptor phosphorylation in rat cerebellum. The greater CB_1 mRNA expression level in female cerebellum is more vulnerable than that of males after exposure to repeated stress. The rapid CB_1 receptor phosphorylation in cerebellum after stress could also be a biomarker for increased anxiety in females after exposure to repeated traumatic stress. If replicated, these findings could help to design gender-specific pharmacological interventions for stress-induced anxiety disorders.

Conflict of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.05.013.

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