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Chapter 4

DELAYED EFFECTS OF REPEATED INESCAPABLE SEVERE STRESS ON BRAIN CANNABINOID RECEPTOR EXPRESSION AND ACOUSTIC STARTLE RESPONSE IN ADOLESCENT MALE RATS: RELEVANCE TO THE DEVELOPMENT OF POSTTRAUMATIC STRESS DISORDER AND STRESS-RELATED BRAIN ATROPHY*

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ABSTRACT

Background: Abnormal brain cannabinoid receptor (CBR, CB_1 and CB_2) activities have been implicated in post-traumatic stress disorder (PTSD) and stress-related psychiatric syndromes. We recently reported altered cannabinoid receptor expression in the cerebellum and brain stem of female animals immediately after 3 days repeated severe stress that was absent in the male animals. We hypothesize that this absence of change of CBR in male brain may reflect a delay rather than a lack of stress effect on CBR expression in male animals.

Methods: Male adolescent rats were exposed to three-day sessions of 2h repeated restraint/tail shock stress daily before being tested twice for behavioral change in acoustic startle response (ASR): immediately after and seven days after the cessation of the last session of stress. CB_1 and CB_2 mRNA expression levels were quantified in the prefrontal cortex, amygdala, hypothalamus, hippocampus, cerebellum and brain stem of adolescent male rats using real-time PCR. CB_1 and CB_2 protein expression levels in the prefrontal cortex, olfactory, cerebellum and brain stem were determined using Western blot.

Key findings: Different from the lack of change in brain CBR expression immediate after the stress in male animals as reported previously, we found significant changes in brain CBR expression7 days after cessation of the stress protocol. Compared to the controls, CB_1 mRNA expression decreased significantly in the prefrontal cortex and brain stem ($P<0.01$), decreased at a trend level in the hippocampus and hypothalamus ($P<0.1$), showed no change in the cerebellum, but increased in the amygdala ($P<0.05$) of stressed male rats. CB_2 mRNA expression decreased significantly in the prefrontal cortex, hypothalamus and brain stem ($P<0.05$), decreased at a trend level in the amygdala and hippocampus ($P<0.1$), and increased in the cerebellum ($P<0.05$) of stressed animals. CB_1 protein expression level and glycosylated CB_1 protein level decreased in the cerebellum but increased in the prefrontal cortex of stressed animals ($p<0.05$, each). CB_2 protein level, however, decreased significantly in the prefrontal cortex ($P<0.01$), cerebellum ($P<0.05$) and brain stem ($P<0.05$) of the stressed animals. These delayed changes in brain CBR expression levels in male animals paralleled with the transition from a hypo ASR immediately after the stress to a hyper ASR 7 days after the cessation of the stress.

Significance: PTSD is a chronic anxiety disorder with a significant period of incubation after trauma exposure and greater prevalence and symptom severity in the females. Different from the previous findings of no change in the cannabinoid system immediately after stress in male animals, significant changes were found in brain cannabinoid receptor expression in male animals seven days after the stress. Importantly, the delayed change in brain CBR appears to parallel with the emergence of enhanced ASR in these animals, suggesting that altered cannabinoid system may contribute significantly to the development of enhanced ASR, a characteristic feature of patients with PTSD. As CB_1 is a potent anxiolytic regulator, the divergent and time-dependent effects of repeated stress on male and female CB_1 expression could contribute to sex dimorphism in PTSD development and symptom severity. In summary, repeated stress caused a delayed effect on brain CBR expression in male animals relative to that in female animals and that could underlie the gender difference in stress-induced anxiety disorders and PTSD.

INTRODUCTION

Cannabinoid receptors are among the most abundant G protein-coupled receptors expressed in the brain and play an important role in fear memory and extinction, emotional behavior, neuroprotection and apoptosis. Abnormal brain cannabinoid receptor expression/activity has been implicated in the pathophysiology of post-traumatic stress disorder (PTSD) and other stress-related psychiatric syndromes. CB_1 knockout animals show hypersensitivity to stressful stimuli, decreased sociability, increased aggressive and anxiety-like behaviors and disrupted response to anxiolytic drugs [21, 31, 54]. CB_1 knockout animals and CB_1 antagonist-treated animals also show marked inability to extinguish fear memory [30]. These characteristics parallel the core clinical features of individuals with 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[24, 38, 57].

Gender differences in PTSD and other stress-related psychological disorders have been documented in the literature, with females showing 2 to 3 fold higher prevalence and more severe symptoms of anxiety and depression than males [8, 9, 27, 39, 45]. However, the mechanism is unknown. We recently reported significant alterations of cannabinoid receptor expression and phosphorylation in the cerebellum and brain stem of female rats immediately after three-day restraint/tail shock [60]. Such alterations, however, were not

observed in the stressed male animals. Since male animals also develop PTSD and other stress-related psychiatric syndromes after stress exposure albeit at a lower prevalence, it may be necessary to look at the prolonged effects of stress on male animals.

In this study, we examined CB_1 and CB_2 expression levels in multiple brain regions of adolescent male rats seven days after the cessation of three-day repeated restraint/ tail shock. Adolescent animals were used because puberty is marked by a substantial increase in many stress-related psychological and physiological disorders (e.g. depression, anxiety, PTSD, and drug abuse)[48], and because a majority of PTSD patients did experience early life stress. Thus, studying adolescent animals could help to determine the initiating mechanisms associated with the development of anxiety disorders.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley (Taconic Farms, Germantown, NY, USA) weighing 120–150 g (five to six weeks old) were used in this study. Animals were housed two per cage and maintained at room temperature (22±2^{o}C) on a 12 hour light-dark schedule (lights on 1800 h). Animals had *ad libitum* access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Uniformed Services University of the Health Sciences, and were carried out in an accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Stress Protocol

Animals were left undisturbed for 7 days after arrival. The stress procedure consisted of a 2-hour per day session of immobilization and tail-shocks over 3 consecutive days. Half of the animals (8 per group) were restrained in a plexiglas tube and given 40 electric tail shocks (2mA, 3 second duration) at varying intervals (140 to 180 seconds). The control animals were handled daily for the same time period but not subjected to the immobilization and tail-shock stress procedures. All animals were returned to their home cages immediately after exposure to the stress or control conditions.

Acoustic Startle Measurement

Acoustic startle response (ASR) measurement (Janusz W. Blaszczyk, Acta Neurobiol. Exp. 2003) was conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, Columbus, Ohio, USA). This system consists of weight-sensitive platforms in a sound-attenuated chamber. The pressures against the platform due to the animals' movements in response to sound stimuli were measured (detail see Jiang, XL et al). In current study the responses to 100 dB sound stimuli were presented. The maximum values were collected in the results and finally adjusted with the animal body weight of the same day to avoid the pressure difference due to different animal body weight on the platform. Animals were tested one day before stress as baseline reading and immediately (day 0) and seven days after the cessation of three-day restraint/tail shock.

Tissue Dissection

Seven days after the last session of the stress, both the control animals and the stressed animals were decapitated after light anesthesia with halothane. The brains were rapidly removed. A Vibratome (Technical Products International, St. Louis, MO, USA) was used to cut 1.6 mm-thick transverse slices containing the whole amygdala region (Bregma -3.60 mm to -2.00 mm) from tissue blocks. The basolateral complex, composed mainly of the lateral and basolateral nuclei, was dissected from this slice laterally, as outlined by the white matter tract of the external capsule (corpus callosum) and medially by the white matter tract of the longitudinal association bundle. This transverse slice (Bregma -3.60 mm to -2.00 mm) also contained the hippocampal dentate gyrus and CA1-CA3 regions as well as part of the hypothalamus. The prefrontal cortex, olfactory, cerebellum and brain stem were similarly dissected. All tissue samples were immediately stored in pre-cooled isopentine (–40^{o}C).

Reverse Transcription and Quantitative Real-time PCR

Dissected brain tissue samples were homogenized and total RNA was extracted using RNeasy kit (Qiagen, Germany) according to the manufacturer’s protocol. One microgram (1 μg) of total RNA was reverse

transcribed into first-strand cDNA using the RETROscript reverse transcriptase kit (Ambion, TX) according to the manufacturer's recommendations.

Fifty nanograms (50 ng) of the reverse transcribed RNA from the RT-reaction was used as the template for quantitative real-time PCR reaction with a final PCR reaction volume of 25 µl and the final concentration of the 5' and 3' PCR primers at 100 nM each. CB1 (TTTCCCACTCATTGACGAGAC, GTGAGCCTTCCAGAGAATGT) and CB2 (AAAGCACACCAACAT GTAGCC, GGAACCAGCATATGAGCAGAA) qPCR primers were designed using Primer3 software (MIT, MA) with the size of amplified cDNA ranging between 90 to150 base pairs [60]. Quantification of CB_1 and CB_2 mRNA expression was performed (in triplicate) using a 2-step PCR reaction procedure on an iQ5 Real-Time PCR System (BioRad, CA) using the SYBR Green SuperMix (BioRad, CA). After initial denaturation at 95^{o}C for 3 minutes, 40 cycles of primer annealing and elongation were conducted at 60^{o}C for 45 seconds, followed by denaturation at 95^{o}C for 10 seconds. Fluorescent emission data was captured, and mRNA levels were quantified using the threshold cycle value (Ct).

Fold change in mRNA expression was calculated using the following equation: Fold=$2^{(\text{CT control -CT stress})}$. To compensate for potential variations in input RNA amounts and the efficiency of reverse transcription, data for CB_1 and CB_2 mRNA of each sample was additionally normalized by reference to the data obtained from house keeping genes β-actin (GenBank accession no. X62085) determined from the same sample. The fold change in the compensated mRNA expression data was calculated using the equation: fold change=$2^{-\Delta\Delta Ct,}$ where ΔCt=target gene Ct –housekeeping gene (β-actin) Ct, and ΔΔCt is ΔCt control - ΔCt stress (or fold change) =$2^{\wedge(\Delta\text{CT control} - \Delta\text{CT stress})}$.

Western Blot

Brain tissues of prefrontal cortex, cerebellum, brainstem and olfactory were homogenized and sonicated for 40 seconds in the T-Per tissue lysis buffer for western blot analysis [Pierce, IL]. Other tissue proteins were not examined due to the limited amount of tissues. Protein concentrations were determined using a Bradford assay (BioRad, CA, USA). Aliquots of 20 µg proteins were separated by electrophoresis on NuPage gels (10%) and transferred to a polyvinylidene difluoride membrane before being incubated with the primary antibodies of CB_1, glycosylated-CB_1, and CB_2 diluted at

1:500, each (Santa Cruze Biotechnologies, CA). The specificity of the CB_1 and CB_2 antibodies were validated by Santa Cruze Biotech and by different research groups [12, 19, 20, 51, 61]. The membranes were rinsed in a 0.01 M Tris-buffered saline solution (pH 7.4) containing 0.1% Triton X-100 for 30 minutes, blocked in 5% non-fat dry milk for 30 minutes and incubated overnight at 4°C with the primary antibody in a Tris-buffered saline solution containing 3% non-fat dry milk. Membranes were washed three times with the Tris-buffered saline solution and incubated overnight at 4° C with a horseradish peroxidase-conjugated secondary antibody in the Tris-buffered saline solution containing 3% non-fat dry milk. Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit antibodies in a 1:3,000 ratio, and ECL Western blotting detection reagents (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The western blots were captured with a digital camera and the intensities quantified with NIH Image 1.62.

STATISTICS

Data regarding the effects of stress on CB_1 and CB_2 receptors for each brain region were analyzed using one-way ANOVA. A p-value less than 0.05 was considered statistically significant.

RESULTS

Compared to that of the controls, CB_1 mRNA expression decreased significantly in the prefrontal cortex and brain stem ($P<0.01$) and decreased at a trend level in the hippocampus ($P<0.1$) and increased significantly in the amygdala ($P<0.05$) of the stressed animals, but it did not change significantly in the cerebellum and hypothalamus (Figure 1). CB_2 mRNA expression decreased significantly in the prefrontal cortex, hypothalamus and brain stem ($P<0.05$), decreased at a trend level in the amygdala and hippocampus ($P<0.1$) but significantly increased in the cerebellum ($P<0.05$) in stressed rats (Figure 1). Total CB_1 protein expression and glycosylated CB_1 protein levels decreased significantly in the cerebellum but increased in the prefrontal cortex of stressed animals ($p<0.05$, each) (Figures 2-3). CB_2 protein level decreased significantly in the prefrontal cortex ($P<0.01$), cerebellum ($P<0.05$) and brain stem ($P<0.05$) 7 days after repeated stress (Figures 2-3).

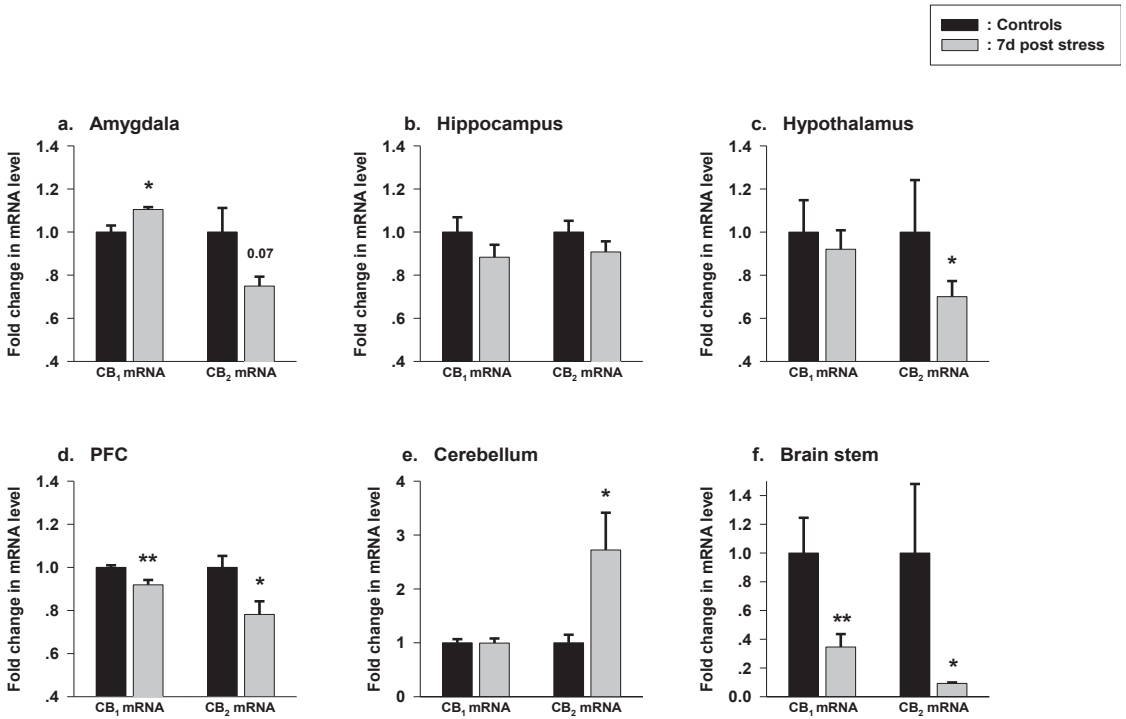

Figure 1. One-way ANOVA of the delayed effect (7days after the stress) of three-day restraint/tail shock on CB_1 and CB_2 mRNA expression levels in the amygdala, a), hippocampus, b), hypothalamus, c), prefrontal cortex, d), cerebellum, e) and brain stem, f) of adolescent male rats. Black column: control group; grey column, stressed group, (n= 8, each group) * $p < 0.05$; ** $p < 0.01$, control vs. stress.

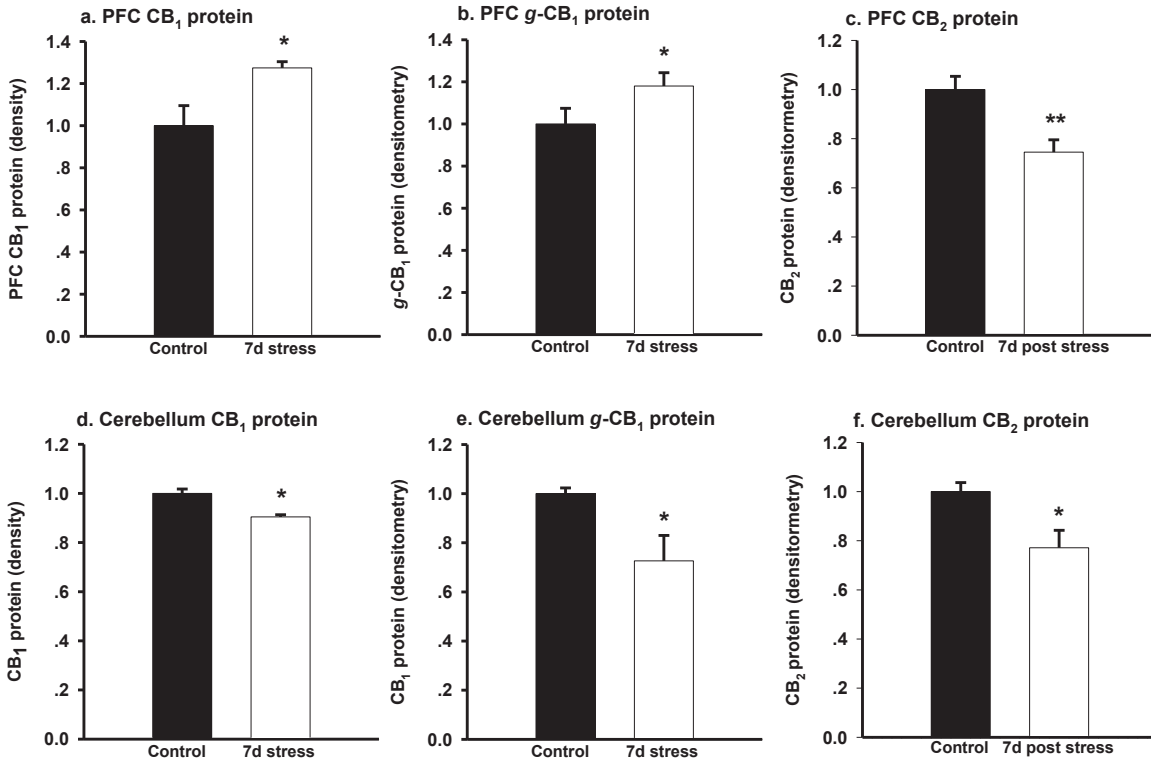

Figure 2. One-way ANOVA of the delayed effect (7days after the stress) of three-day restraint/tail shock on CB_1 and CB_2 protein expression levels in the prefrontal cortex, a), and cerebellum, b) of adolescent male rats. Black column: control group; blank column, stressed group, (n= 8, each group) * $p < 0.05$; ** $p < 0.01$, control vs. stress.

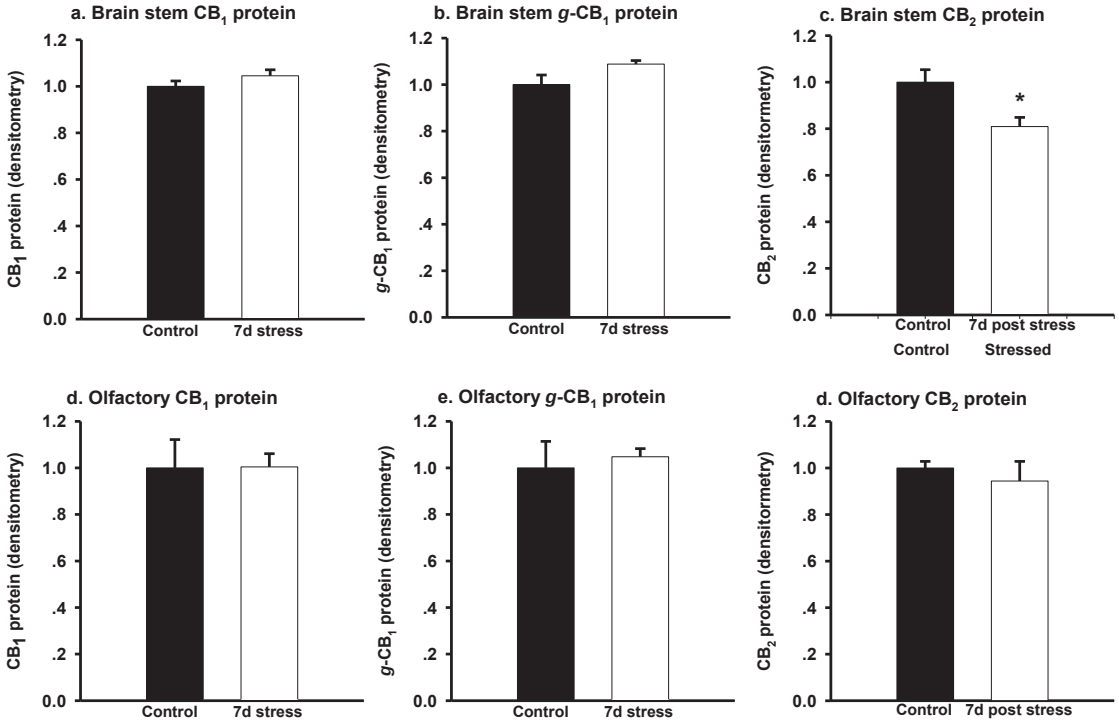

Figure 3. O One-way ANOVA of the delayed effect (7days after the stress) of three-day restraint/tail shock on CB_1 and CB_2 protein expression levels in the brainstem, a), and olfactory, b) of adolescent male rats. Black column: control group; blank column, stressed group, (n= 8, each group), * $p < 0.05$, control vs. stress.

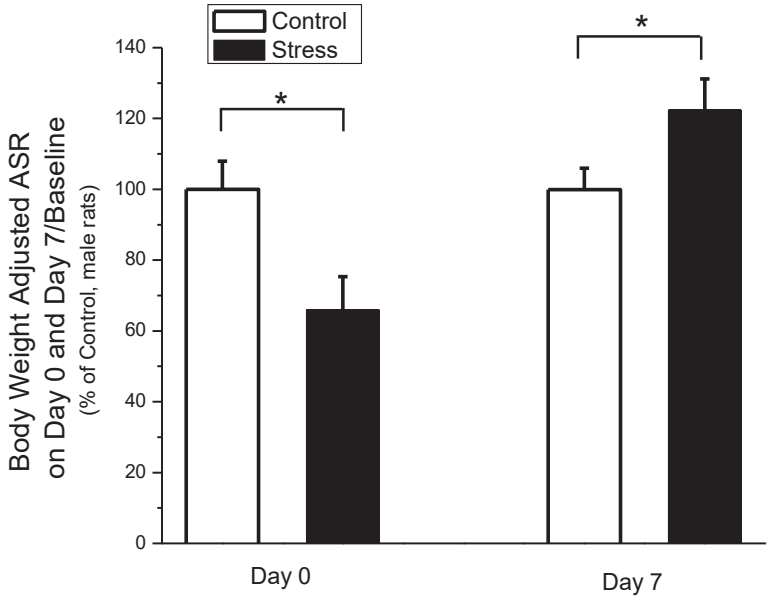

Figure 4. Immediate and delayed effects of three-day restraint/tail shock on peak value of acoustic startle response (ASR) (adjusted with same day body weight of each animal) in adolescent male rats) immediately after three day stress versus that of the baseline value in stress group in the stressed animals when compared with that of the control group (as 100%, $p<0.05$). (Mean ± S.E.M). It is noticed that , this peak value of ASR increased significantly in the stressed male animals 7 days after the cessation of three-day restraint/tail shock compared with that of the control group ($p<0.05$). * $p < 0.05$, control vs. stress, (N= 24, for each of the control and stress group for Day 0, and N=16, each group for Day 7).

A significant reduction in acoustic startle response (ASR) to 100 dB of sound stimuli (adjusted with same day body weight of each animal) immediately after three-day restraint/tail shock occurred in the stressed animals compared with that of the control group (as 100%, $p<0.05$) (Figure 4).

However, acoustic startle response increased significantly in the stressed animals 7 days after the cessation of three-day restraint/tail shock compared with the control group ($p<0.05$) (Figure 4).

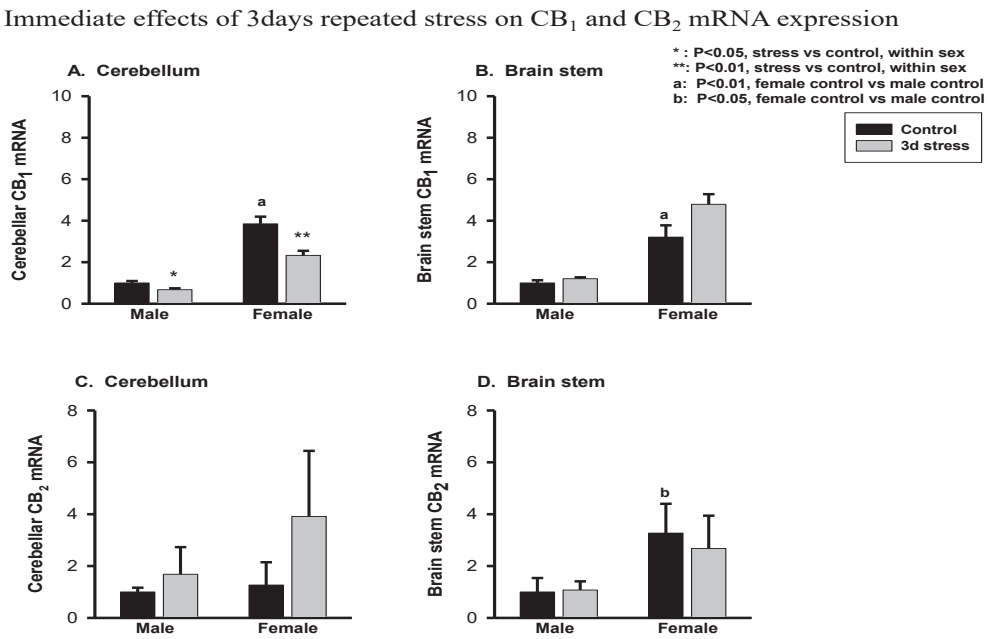

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Figure 5. Two-way ANOVA of the immediate effects of three-day restraint/tail shock CB_1 and CB_2 mRNA expression levels in the cerebellum, a) and c) , and brain stem, b) and d) of adolescent male and female rats. Black column: control rats; grey column, stressed rats, (n= 8, each group), a, $p<0.01$, male control vs. female control; b, $p<0.05$, male control vs. female control; *, $p<0.05$; **, $p<0.01$, control vs. stress, within-sex 1-way ANOVA.

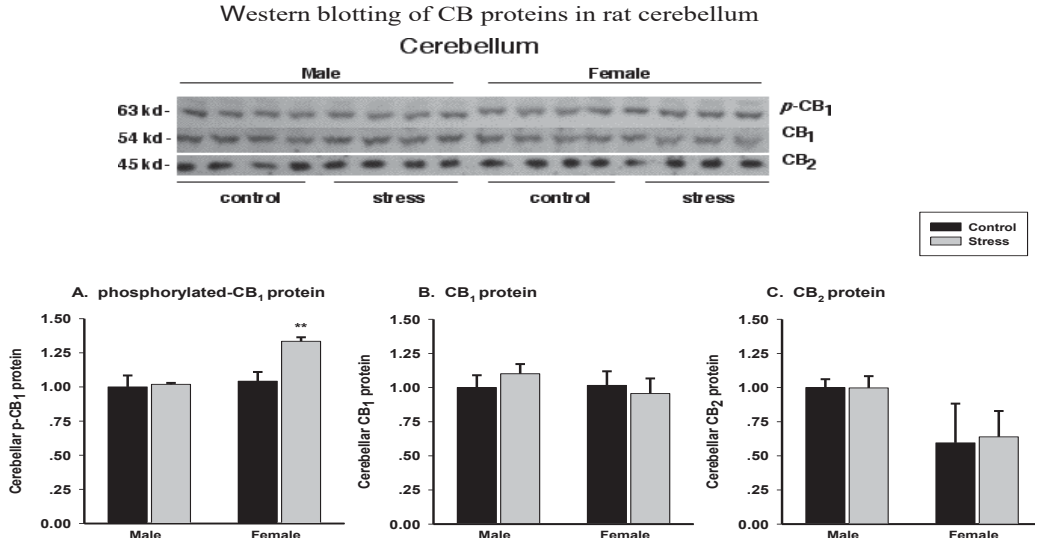

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Figure 6. Two-way ANOVA of the immediate effects of three-day restraint/tail shock on CB_1 and CB_2 protein expression levels in the cerebellum. *Top panel,* representatives of cerebellum western blotting: twenty micrograms (μg) of rat cerebellum tissue homogenates were resolved on SDS-PAGE gel and incubated with the specific CB antibodies and detected using an ECL detecting system. *Bottom panel,* semi-quantitative Western blotting of A), phosphorylated CB_1 proteins (p-CB1); B), total CB_1 proteins; C), CB_2 proteins in cerebellar tissue homogenates of adolescent male and female rats. 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-CB1 protein in the stressed animals, primarily in the females*, $p<0.05$; Black bar, control group; gray bar, stress group (n=8, each group).

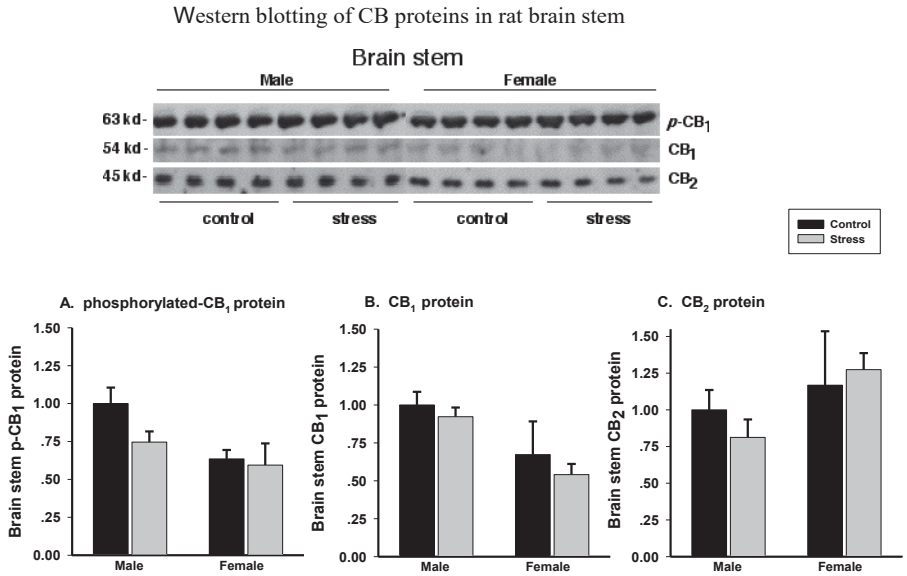

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Figure 7. Two-way ANOVA of the immediate effects of three-day restraint/tail shock on CB_1 and CB_2 protein expression levels in brain stem. *Top panel,* representatives of brain stem western blotting: twenty micrograms (μg) of rat tissue homogenates were resolved on SDS-PAGE gel and incubated with the specific CB antibodies and detected using an ECL detecting system. *Bottom panel,* semi-quantitative Western blotting of A), phosphorylated CB_1 proteins (p-CB1); B), total CB_1 proteins; C), CB_2 proteins in the brain stem of adolescent male and female rats. 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 bar, control group; gray bar, stress group (n=8, each group).

DISCUSSION

In this study, CB_1 mRNA expression decreased significantly in the prefrontal cortex and brain stem, decreased at trend level in the hippocampus and hypothalamus, but increased significantly in the amygdala of male animals 7 days after the repeated stress. This result, while in contrast to the lack of brain CB_1 mRNA alteration in the male animals immediately after the stress (Figure 5-7) [60], supports the notion that the cannabinoid receptor in the male animals is also vulnerable to the detrimental effect of repeated severe stress, albeit in a delayed manner when compared with the female animals.

Repeated stress also reduced CB_2 mRNA expression in most brain regions except cerebellum where CB_2 mRNA levels increased 7 days after the stress. Such delayed alteration of CB_2 mRNA expression was not observed in the brain of male and female animals immediately after the stress [60] (data submitted).

Discordance between CB mRNA and protein expression is noticed in this study: stress reduced CB_1 mRNA level of the PFC and brain stem but increased CB_1 protein level in the PFC and no change in the brain stem; stress reduced cerebellar CB_1 and CB_2 protein levels but increased cerebellar CB_2 mRNA level and no change in CB_1 mRNA level. While no easy explanation is available, this may reflect different induction and degradation mechanisms between the mRNA and protein. The induction of protein expression is usually slow and lags behind that of mRNA which often has a shorter half-life than proteins. The greater CB_1 protein level in the prefrontal cortex of the stressed male rats may be transient if the reduced prefrontal CB_1 mRNA level is an indication of further change in brain CB_1 protein expression in the male animals. A recent study showed reduced CB_1 receptor protein levels in the putamen and the nucleus accumbens of young male rats after 8 weeks isolation rearing stress [28]. Three weeks of chronic mild stress is also effective in reducing CB_1 receptor proteins in the hippocampus of adult male rats [46].

PTSD is a chronic anxiety disorder with a significant incubating time period after the trauma exposure. However, the underlying mechanism remains unknown. Our study suggests that a delayed alteration of brain cannabinoid receptor expression could be involved. Indeed, the delayed CB_1 receptor alteration coincides with the progressive increase in acoustic startle response in the stressed male animals [50].

The data from our studies indicate that male and female adolescent animals differ significantly in the prefrontal cortex response to the repeated stress as reflected in the divergent prefrontal CB_1 protein expression after the

stress (data submitted for publication). The adolescent male animals show a trend of increased CB_1 protein expression in the prefrontal cortex immediately after the stress that become significant at 7 days after the stress whereas the female rats show significantly reduced prefrontal CB_1 receptor expression immediately after the stress (data submitted for publication).

Prefrontal cortex is known to exert a powerful inhibitory effect on amygdala activity and plays an important role in fear extinction [25, 59]. The initial increase in CB_1 expression and CB_1–mediated signaling in the prefrontal cortex of adolescent male animals immediately after the stress could potentially inhibit amygdala activation, control fear memory and facilitate the reorganization of the interconnection and inter-regulation between the regions to strength the endurance and resistance to repeated stress in the male animals. Whereas the rapidly reduced CB_1 expression in the prefrontal cortex of female animals could potentially weaken the inhibitory effect of prefrontal cortex on amygdala activity, and cause increased fear and a greater emotional disturbance in the stressed female animals. Further studies are warranted to test these possibilities.

It should be noted that the increased CB_1 protein in the prefrontal cortex of male animals could be transient and short-lived especially if the intensity and the duration are strong and long enough as reflected in the reduced CB_1 mRNA expression 7 days after the completion of the stress that could predict a subsequent reduction of CB_1 protein expression in the prefrontal cortex of stressed male animals. This, again needs to be verified in future studies.

The divergently induced CB_1 receptor expression in the prefrontal cortex of male and female animals by stress could play an important role in the gender difference in the prevalence and severity of anxiety disorders, PTSD and stress-related psychological symptoms, by favoring increased stress endurance and fear control in the males and by increased emotional disturbance and pain in the females [23, 34]. Forebrain CB_1 receptor activation is known to exert anxiolytic function [10, 41, 43] whereas loss or inhibition of CB_1 receptor in the amygdala, hippocampus, and prefrontal cortex is associated with inability to extinguish fear memory [14, 30, 47].

In this study, CB_2 receptor protein levels decreased significantly in the prefrontal cortex of male rats 7 days after repeated stress. Similar reduction of CB_2 receptor levels were found in the prefrontal cortex of male and female rats immediately after the stress (data submitted). The biological implication of the stress-induced deficiency in CB_2 expression remains to be understood.

One possibility is that CBR deficiency could be involved in the reduced volumes of prefrontal cortex as well as reduced hippocampus and cerebellum

volumes in subjects with PTSD and with traumatic stress exposure [13, 18, 58, 63]. CB_1 and CB_2 receptor exert neuroprotective functions through multi anti-inflammation, anti-apoptosis and pro-neurogenesis mechanisms [11, 29, 42, 53, 62]. CB_1 and CB_2 also regulate neural progenitor (NP) cell proliferation and neurogenesis. CB_1 receptor activation promotes NP cell proliferation and neurosphere generation in postnatal and adult brain, an action that is abrogated in CB_1-deficient multipotent NP cells. CB_2 receptor activation also induced cell proliferation in embryonic cortical slices and in adult hippocampal NPs [1, 2, 40].

The significantly reduced cannabinoid receptor mRNA and protein expression in the brain stem of the stressed animal is consistent with the fact that periaqueductal gray, locus coeruleus (LC), raphe nuclei, reticular activating system, solitary tract, vestibular and cochlear nuclei embedded in the brain stem are involved in the regulation of pain sensation, sleep-awake cycle, acoustic startle response, cardiovascular reactivity that are frequently altered in subjects with PTSD [3, 4, 15, 33, 35, 36, 44, 56]. Further immunohistochemistry/*in situ* hybridization studies are required to determine if and how changes of cannabinoid receptor expression in this brain stem nucleus are correlated with the enhanced acoustic startle response, increased cardiovascular and sleep disorder and pain in PTSD.

Since stress enhances 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. Alternatively, CB 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 [49]. Thus, brain CB and adrenergic systems could interact in central regulation of cardiovascular response in stress-induced anxiety disorders [7].

In this study, CB_1 and CB_2 receptor protein level decreased significantly in the cerebellum of male rats 7 days after the repeated stress. Reduced CB_1 receptor expression was also found in the cerebellum but not brain stem of adolescent female rats immediately after the stress (Figures 4 and 6) [60]. A recent study showed that acute postnatal maternal deprivation stress significantly increased neuronal apoptosis in the cerebellum of male rat pups and that increased apoptosis was attenuated by inhibiting endogenous cannabinoid (eCBs) degradation [26], suggesting an important neuroprotective role of eCBs in cerebellum. Furthermore, significant correlations were also found between structural volumes of cerebellum and the clinical symptoms of

pediatric maltreatment-related PTSD, with PTSD subjects had significantly reduced volume of the left and right hemispheres and total cerebellum volume [13]. These data show a close link between stress-induced apoptosis and cerebellum atrophy in PTSD although the exact mediator(s) and the time course of the atrophy remain unknown.

Our present study suggests that repeated stress-induced CB_1 receptor reduction in adolescence could be involved in the cerebellar neuronal apoptosis and structural atrophy in PTSD.

Despite the highest expression of cannabinoid receptors (CB_1 and CB_2) in the cerebellum [20, 25, 48], its functional role in PTSD remains to be established. The downregulation of cerebellar CB_1 receptor after repeated stress could have contributed to altered time sense in PTSD and cerebellar cannabinoid hypofunction in subjects of frequent marijuana use [55]. Impaired time sense has been reported in marijuana users who exhibited cerebellar hypoactivity in response to delta-9-tetrahydrocannabinol (a potent CB_1 agonist and primary component of marijuana) [32].

As marijuana use is common among PTSD subjects and is correlated with the severity of PTSD symptoms [5], our studies imply that such increased marijuana use in subjects with PTSD and with traumatic exposure may be a compensatory mechanism or self-medicine to counter-balance the stress-induced brain deficiency of CB receptor expression and activity [6, 9, 16, 17, 22, 37, 52].

CONCLUSION

We have demonstrated that repeated inescapable intense stress can indeed induce delayed but significant alterations in brain CB_1 and CB_2 receptor expression of the adolescent male animals albeit absent immediately after the stress. Such delayed alteration when compared with the immediate alteration in the female animals may underlie the gender difference in the prevalence and severity of stress-related anxiety disorders including PTSD. Because of the potent anxiolytic, neuroprotective, neural progenitor cell proliferating, and neurosphere generating effects of CB_1 and CB_2 , further studies of CB_1 and CB_2 in stressed animals could lead to better understanding and treatment of PTSD and stress-related psychological symptoms.

DISCLOSURE/CONFLICT OF INTEREST

All authors declare no conflict of interest.

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REFERENCES

[1] T. Aguado, K. Monory, J. Palazuelos, N. Stella, B. Cravatt, B. Lutz, G. Marsicano, Z. Kokaia, M. Guzman, I. Galve-Roperh, The endocannabinoid system drives neural progenitor proliferation, *Faseb J.* 19 (2005) 1704-1706.

[2] T. Aguado, J. Palazuelos, K. Monory, N. Stella, B. Cravatt, B. Lutz, G. Marsicano, Z. Kokaia, M. Guzman, I. Galve-Roperh, The endocannabinoid system promotes astroglial differentiation by acting on neural progenitor cells, *J. Neurosci*. 26 (2006) 1551-1561.

[3] G.J. Asmundson, M.J. Coons, S. Taylor, J. Katz, PTSD and the experience of pain: research and clinical implications of shared vulnerability and mutual maintenance models, *Can. J. Psychiatry* 47 (2002) 930-937.

[4] G.J. Asmundson, J. Katz, Understanding pain and posttraumatic stress disorder comorbidity: do pathological responses to trauma alter the perception of pain?, *Pain* 138 (2008) 247-249.

[5] M.O. Bonn-Miller, A.A. Vujanovic, M.T. Feldner, A. Bernstein, M.J. Zvolensky, Posttraumatic stress symptom severity predicts marijuana use coping motives among traumatic event-exposed marijuana users, *J. Trauma Stress* 20 (2007) 577-586.

[6] M.A. Bornovalova, P. Ouimette, A.V. Crawford, R. Levy, Testing gender effects on the mechanisms explaining the association between post-traumatic stress symptoms and substance use frequency, *Addict. Behav*. 34 (2009) 685-692.

[7] M.F. Braga, V. Aroniadou-Anderjaska, S.T. Manion, C.J. Hough, H. Li, Stress impairs alpha(1A) adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala, *Neuropsychopharmacology* 29 (2004) 45-58.

[8] N. Breslau, The epidemiology of trauma, PTSD, and other posttrauma disorders, *Trauma Violence Abuse* 10 (2009) 198-210.

[9] N. Breslau, H.D. Chilcoat, R.C. Kessler, E.L. Peterson, V.C. Lucia, Vulnerability to assaultive violence: further specification of the sex difference in post-traumatic stress disorder, *Psychol. Med*. 29 (1999) 813-821.

[10] J.P. Chhatwal, M. Davis, K.A. Maguschak, K.J. Ressler, Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear, *Neuropsychopharmacology* 30 (2005) 516-524.

[11] B. Czeh, J.I. Muller-Keuker, R. Rygula, N. Abumaria, C. Hiemke, E. Domenici, E. Fuchs, Chronic social stress inhibits cell proliferation in the adult medial prefrontal cortex: hemispheric asymmetry and reversal by fluoxetine treatment, *Neuropsychopharmacology* 32 (2007) 1490-1503.

[12] G. Czifra, A. Varga, K. Nyeste, R. Marincsak, B.I. Toth, I. Kovacs, L. Kovacs, T. Biro, Increased expressions of cannabinoid receptor-1 and transient receptor potential vanilloid-1 in human prostate carcinoma, *J. Cancer Res. Clin. Oncol*. 135 (2009) 507-514.

[13] M.D. De Bellis, M. Kuchibhatla, Cerebellar volumes in pediatric maltreatment-related posttraumatic stress disorder, *Biol. Psychiatry* 60 (2006) 697-703.

[14] L. De Oliveira Alvares, B.P. Genro, F. Diehl, J.A. Quillfeldt, Differential role of the hippocampal endocannabinoid system in the memory consolidation and retrieval mechanisms, *Neurobiol. Learn Mem*. 90 (2008) 1-9.

[15] A. Dogrul, L.R. Gardell, S. Ma, M.H. Ossipov, F. Porreca, J. Lai, 'Knock-down' of spinal CB1 receptors produces abnormal pain and elevates spinal dynorphin content in mice, *Pain* 100 (2002) 203-209.

[16] M. Driessen, S. Schulte, C. Luedecke, I. Schaefer, F. Sutmann, M. Ohlmeier, U. Kemper, G. Koesters, C. Chodzinski, U. Schneider, T. Broese, C. Dette, U. Havemann-Reinicke, Trauma and PTSD in patients

with alcohol, drug, or dual dependence: a multi-center study, *Alcohol. Clin. Exp. Res.* 32 (2008) 481-488.

[17] L. Fattore, M.S. Spano, S. Deiana, V. Melis, G. Cossu, P. Fadda, W. Fratta, An endocannabinoid mechanism in relapse to drug seeking: a review of animal studies and clinical perspectives, *Brain Res. Rev*. 53 (2007) 1-16.

[18] K. Felmingham, L.M. Williams, T.J. Whitford, E. Falconer, A.H. Kemp, A. Peduto, R.A. Bryant, Duration of posttraumatic stress disorder predicts hippocampal grey matter loss, *Neuroreport* 20 (2009) 1402-1406.

[19] B.M. Fonseca, G. Correia-da-Silva, A.H. Taylor, J.C. Konje, S.C. Bell, N.A. Teixeira, Spatio-temporal expression patterns of anandamide-binding receptors in rat implantation sites: evidence for a role of the endocannabinoid system during the period of placental development, *Reprod. Biol. Endocrinol*. 7 (2009) 121.

[20] D.E. Garcia, S. Brown, B. Hille, K. Mackie, Protein kinase C disrupts cannabinoid actions by phosphorylation of the CB1 cannabinoid receptor, *J. Neurosci*. 18 (1998) 2834-2841.

[21] J. Haller, N. Bakos, M. Szirmay, C. Ledent, T.F. Freund, The effects of genetic and pharmacological blockade of the CB1 cannabinoid receptor on anxiety, *Eur. J. Neurosci.* 16 (2002) 1395-1398.

[22] C.A. Hernandez-Avila, B.J. Rounsaville, H.R. Kranzler, Opioid-, cannabis- and alcohol-dependent women show more rapid progression to substance abuse treatment, *Drug Alcohol. Depend*. 74 (2004) 265-272.

[23] M.M. Kelly, A.R. Tyrka, G.M. Anderson, L.H. Price, L.L. Carpenter, Sex differences in emotional and physiological responses to the Trier Social Stress Test, *J. Behav. Ther. Exp. Psychiatry* 39 (2008) 87-98.

[24] K.C. Koenen, M.J. Lyons, J. Goldberg, J. Simpson, W.M. Williams, R. Toomey, S.A. Eisen, W. True, M.T. Tsuang, Co-twin control study of relationships among combat exposure, combat-related PTSD, and other mental disorders, *J. Trauma Stress* 16 (2003) 433-438.

[25] C.S. Li, R. Sinha, Inhibitory control and emotional stress regulation: neuroimaging evidence for frontal-limbic dysfunction in psycho-stimulant addiction, *Neurosci. Biobehav. Rev*. 32 (2008) 581-597.

[26] M. Lopez-Gallardo, R. Llorente, A. Llorente-Berzal, E.M. Marco, C. Prada, V. Di Marzo, M.P. Viveros, Neuronal and glial alterations in the cerebellar cortex of maternally deprived rats: gender differences and modulatory effects of two inhibitors of endocannabinoid inactivation, *Dev. Neurobiol*. 68 (2008) 1429-1440.

[27] D.D. Luxton, N.A. Skopp, S. Maguen, Gender differences in depression and PTSD symptoms following combat exposure, *Depress Anxiety* 27 (2010) 1027-1033.

[28] D.T. Malone, C.S. Kearn, L. Chongue, K. Mackie, D.A. Taylor, Effect of social isolation on CB1 and D2 receptor and fatty acid amide hydrolase expression in rats, *Neuroscience* 152 (2008) 265-272.

[29] Y. Marchalant, H.M. Brothers, G.J. Norman, K. Karelina, A.C. DeVries, G.L. Wenk, Cannabinoids attenuate the effects of aging upon neuroinflammation and neurogenesis, *Neurobiol. Dis.* 34 (2009) 300-307.

[30] G. Marsicano, C.T. Wotjak, S.C. Azad, T. Bisogno, G. Rammes, M.G. Cascio, H. Hermann, J. Tang, C. Hofmann, W. Zieglgansberger, V. Di Marzo, B. Lutz, The endogenous cannabinoid system controls extinction of aversive memories, *Nature* 418 (2002) 530-534.

[31] M. Martin, C. Ledent, M. Parmentier, R. Maldonado, O. Valverde, Involvement of CB1 cannabinoid receptors in emotional behaviour, *Psychopharmacology* (Berl) 159 (2002) 379-387.

[32] R.J. Mathew, W.H. Wilson, T.G. Turkington, R.E. Coleman, Cerebellar activity and disturbed time sense after THC, *Brain Res*. 797 (1998) 183-189.

[33] T.A. Mellman, A. Kumar, R. Kulick-Bell, M. Kumar, B. Nolan, Nocturnal/daytime urine noradrenergic measures and sleep in combat-related PTSD, *Biol. Psychiatry* 38 (1995) 174-179.

[34] A. Meulders, D. Vansteenwegen, J.W. Vlaeyen, Women, but not men, report increasingly more pain during repeated (un)predictable painful electrocutaneous stimulation: evidence for mediation by fear of pain, *Pain* (2012).

[35] C.A. Morgan, 3rd, C. Grillon, H. Lubin, S.M. Southwick, Startle reflex abnormalities in women with sexual assault-related posttraumatic stress disorder, *Am. J. Psychiatry* 154 (1997) 1076-1080.

[36] C.A. Morgan, 3rd, C. Grillon, S.M. Southwick, M. Davis, D.S. Charney, Exaggerated acoustic startle reflex in Gulf War veterans with posttraumatic stress disorder, *Am. J. Psychiatry* 153 (1996) 64-68.

[37] S.E. Nunnink, G. Goldwaser, P.S. Heppner, J.O. Pittman, C.M. Nievergelt, D.G. Baker, Female veterans of the OEF/OIF conflict: concordance of PTSD symptoms and substance misuse, *Addict. Behav*. 35 (2010) 655-659.

[38] G.T. Okulate, O.B. Jones, Post-traumatic stress disorder, survivor guilt and substance use--a study of hospitalised Nigerian army veterans, *S. Afr. Med. J*. 96 (2006) 144-146.

[39] M. Olff, W. Langeland, N. Draijer, B.P. Gersons, Gender differences in posttraumatic stress disorder, *Psychol. Bull*. 133 (2007) 183-204.

[40] J. Palazuelos, Z. Ortega, J. Diaz-Alonso, M. Guzman, I. Galve-Roperh, CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling, *J. Biol. Chem*. 287 (2012) 1198-1209.

[41] F.A. Pamplona, R.M. Bitencourt, R.N. Takahashi, Short- and long-term effects of cannabinoids on the extinction of contextual fear memory in rats, *Neurobiol. Learn Mem.* 90 (2008) 290-293.

[42] D. Panikashvili, R. Mechoulam, S.M. Beni, A. Alexandrovich, E. Shohami, CB1 cannabinoid receptors are involved in neuroprotection via NF-kappa B inhibition, *J. Cereb. Blood Flow Metab.* 25 (2005) 477-484.

[43] S. Patel, C.J. Hillard, Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling, *J. Pharmacol. Exp. Ther*. 318 (2006) 304-311.

[44] N. Pole, T.C. Neylan, C. Otte, C. Henn-Hasse, T.J. Metzler, C.R. Marmar, Prospective prediction of posttraumatic stress disorder symptoms using fear potentiated auditory startle responses, *Biol. Psychiatry* 65 (2009) 235-240.

[45] L.C. Pratchett, M.R. Pelcovitz, R. Yehuda, Trauma and violence: are women the weaker sex?, *Psychiatr. Clin. North Am*. 33 (2010) 465-474.

[46] C.G. Reich, M.E. Taylor, M.M. McCarthy, Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats, *Behav. Brain Res*. 203 (2009) 264-269.

[47] M. Roche, E. O'Connor, C. Diskin, D.P. Finn, The effect of CB(1) receptor antagonism in the right basolateral amygdala on conditioned fear and associated analgesia in rats, *Eur. J. Neurosci*. 26 (2007) 2643-2653.

[48] R.D. Romeo, Pubertal maturation and programming of hypothalamic-pituitary-adrenal reactivity, *Front Neuroendocrinol*. 31 (2010) 232-240.

[49] E. Schlicker, M. Gothert, Interactions between the presynaptic alpha2-autoreceptor and presynaptic inhibitory heteroreceptors on noradrenergic neurones, *Brain Res. Bull*. 47 (1998) 129-132.

[50] R.J. Servatius, J.E. Ottenweller, B.H. Natelson, Delayed startle sensitization distinguishes rats exposed to one or three stress sessions:

further evidence toward an animal model of PTSD, *Biol. Psychiatry* 38 (1995) 539-546.

[51] C. Song, A.C. Howlett, Rat brain cannabinoid receptors are N-linked glycosylated proteins, *Life Sci*. 56 (1995) 1983-1989.

[52] T.P. Sullivan, L.J. Holt, PTSD symptom clusters are differentially related to substance use among community women exposed to intimate partner violence, *J. Trauma Stress* 21 (2008) 173-180.

[53] A.O. Tishkina, I.P. Levshina, N.A. Lazareva, N.V. Passikova, M.Y. Stepanichev, M.G. Ajrapetyanz, N.V. Gulyaeva, Chronic stress induces nonapoptotic neuronal death in the rat hippocampus, *Dokl. Biol. Sci*. 428 (2009) 403-406.

[54] L. Uriguen, S. Perez-Rial, C. Ledent, T. Palomo, J. Manzanares, Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors, *Neuropharmacology* 46 (2004) 966-973.

[55] R.J. Ursano, C.S. Fullerton, Posttraumatic stress disorder: cerebellar regulation of psychological, interpersonal, and biological responses to trauma?, *Psychiatry* 62 (1999) 325-328.

[56] M.D. Van Sickle, M. Duncan, P.J. Kingsley, A. Mouihate, P. Urbani, K. Mackie, N. Stella, A. Makriyannis, D. Piomelli, J.S. Davison, L.J. Marnett, V. Di Marzo, Q.J. Pittman, K.D. Patel, K.A. Sharkey, Identification and functional characterization of brainstem cannabinoid CB2 receptors, *Science* 310 (2005) 329-332.

[57] S. Vetter, A. Rossegger, W. Rossler, J.I. Bisson, J. Endrass, Exposure to the tsunami disaster, PTSD symptoms and increased substance use - an Internet based survey of male and female residents of Switzerland, BMC Public Health 8 (2008) 92.

[58] G. Villarreal, D.A. Hamilton, H. Petropoulos, I. Driscoll, L.M. Rowland, J.A. Griego, P.W. Kodituwakku, B.L. Hart, R. Escalona, W.M. Brooks, Reduced hippocampal volume and total white matter volume in posttraumatic stress disorder, *Biol. Psychiatry* 52 (2002) 119-125.

[59] I. Vlachos, C. Herry, A. Luthi, A. Aertsen, A. Kumar, Context-dependent encoding of fear and extinction memories in a large-scale network model of the basal amygdala, *PLoS Comput. Biol.* 7 (2011) e1001104.

[60] G. Xing, J. Carlton, L. Zhang, X. Jiang, C. Fullerton, H. Li, R. Ursano, 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, *Neurosci. Lett*. 502 (2011) 5-9.

[61] T.S. Yu, Z.H. Cheng, L.Q. Li, R. Zhao, Y.Y. Fan, Y. Du, W.X. Ma, D.W. Guan, The cannabinoid receptor type 2 is time-dependently expressed during skeletal muscle wound healing in rats, *Int. J. Legal. Med*. 124 (2010) 397-404.

[62] J. Yun, H. Koike, D. Ibi, E. Toth, H. Mizoguchi, A. Nitta, M. Yoneyama, K. Ogita, Y. Yoneda, T. Nabeshima, T. Nagai, K. Yamada, Chronic restraint stress impairs neurogenesis and hippocampus-dependent fear memory in mice: possible involvement of a brain-specific transcription factor Npas4, *J. Neurochem*. 114 (2010) 1840-1851.

[63] J. Zhang, Q. Tan, H. Yin, X. Zhang, Y. Huan, L. Tang, H. Wang, J. Xu, L. Li, Decreased gray matter volume in the left hippocampus and bilateral calcarine cortex in coal mine flood disaster survivors with recent onset PTSD, *Psychiatry Res.* 192 (2011) 84-90.